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Pathways for Fatty Acid Elongation and Desaturation in *Neurospora crassa*

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ABSTRACT: *Neurospora crassa* incorporated exogenous deuterated palmitate (16:0) and ^{14}C-labeled oleate (18:1$^{\Delta 9}$) into cell lipids. Of the exogenous 18:1$^{\Delta 9}$ incorporated, 59% was desaturated to 18:2$^{\Delta 9,12}$ and 18:3$^{\Delta 9,12,15}$. Of the exogenous 16:0 incorporated, 20% was elongated to 18:0, while 37% was elongated and desaturated into 18:1$^{\Delta 9}$, 18:2$^{\Delta 9,12}$, and 18:3$^{\Delta 9,12,15}$. The mass of unsaturated fatty acids in phospholipid and triacylglycerol is 12 times greater than the mass of 18:0. Deuterium label incorporation in unsaturated fatty acids is only twofold greater than in 18:0, indicating a sixfold preferential use of 16:0 for saturated fatty acid synthesis. These results indicate that the release of 16:0 from fatty acid synthase is a key control point that influences fatty acid composition in *Neurospora*.
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The fatty acid composition of lipids determines their structural and functional properties. However, the biochemical control of fatty acid composition is not well understood. The use of model systems has provided considerable insight in elucidating fatty acid and glycerolipid metabolism.

The filamentous fungus *Neurospora crassa* has advantages as a model system for comparison to plants: it is one of few well-studied microbes able to produce the polyunsaturated fatty acids *de novo* that plants also make (1). It is also capable of incorporating exogenous fatty acid into its lipids (2–4). In addition, several mutants with defects in the synthesis of fatty acids and glycerolipids (3–7) have been described, and the lipid composition of wild type and mutants has been examined under varying conditions of temperature, medium, and fatty acid supplementation (2–4,8–10).

In *Neurospora*, as in plants, the pathway for biosynthesis of the major fatty acids begins with formation of 16:0 from acetyl-CoA and malonyl-CoA. In the major pathway of fatty acid biosynthesis, other fatty acids are derived from 16:0: 16:0 is first elongated to stearate (18:0), which undergoes consecutive desaturations to form 18:1$^{\Delta 9}$, 18:2$^{\Delta 9,12}$, and 18:3$^{\Delta 9,12,15}$ (1). We have examined the metabolism of exogenous palmitate (16:0) and oleate (18:1$^{\Delta 9}$) by *Neurospora* to better understand the regulation of fatty acid biosynthesis and control of fatty acid composition.

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EXPERIMENTAL PROCEDURES

Growth of cultures, fatty acid-supplementation conditions, and label incorporation. Neurospora crassa wild-type strain 74-OR8-1a was obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center (Kansas City, KS). Conidia (1×10^5) were inoculated into culture flasks containing 50 mL of liquid Vogel's medium N (5) and incubated at 34°C with shaking for 24 h. The concentration of labeled fatty acids was 30 μM: Deuterated 16:0's [7,7,8,8-$^2H_4$]16:0; [2,2-$^2H_2$]16:0, [16,16,16-$^2H_3$]16:0, all 98% purity, Cambridge Isotope Laboratories (Andover, MA) were not diluted, and [1-^{14}C]18:1$^{\Delta 9}$—86.4 kBq [^{14}C]18:1$^{\Delta 9}$, 1.11-2.22 GBq/mmol, ICN Pharmaceuticals, Inc. (Costa Mesa, CA) was diluted with unlabeled 18:1$^{\Delta 9}$ (Sigma, St. Louis, MO). Cultures were incubated for another 24 h before lipid extraction. Under these conditions, addition of the fatty acid had no apparent effect on rate of growth or final culture weight.

Lipid analysis. Lipids were extracted from cultures, fractionated on Sep-Pak silica columns to yield neutral lipids, phospholipids, and glycolipids; the triacylglycerols were separated from other neutral lipids by thin-layer chromatography as previously described (5). Radiolabel incorporation into lipid fractions was determined by scintillation counting of aliquots.

Fatty acids were analyzed as fatty acid methyl esters by gas chromatography with flame-ionization detection as described (5), using methyl heptadecanoate as internal standard. The column was a fused silica capillary column (30 m × 0.25 mm) coated with Stabilwax (df = 0.2 μm) (Restex, Bellefonte, PA). Label incorporation into fatty acids of ^{14}C-labeled samples was monitored by high-performance liquid chromatography of fatty acid methyl esters as in Reference 11.

Gas chromatography–mass spectrometry (fused silica capillary, 20 m by 0.18 mm, coated with DB-WAX and df = 0.3 μm; J&W Scientific, Folsom, CA) was used to confirm the identity of fatty acid derivatives and to determine the metabolic fate of deuterated 16:0 (5). Identification was based on comparison of spectra from sample runs to those of standards and published spectra.

The metabolic fate of [7,7,8,8-$^2H_4$]16:0 was followed by operating the mass spectrometer in the selected ion monitoring mode to monitor the M^+ and $(M + 4)^+$ ions of fatty acid

methyl esters from C_{14} to C_{24}, as well as the $(M + 2)^+$ ions for C_{18} unsaturated fatty acids (since desaturation at the 9,10-position following elongation to 18:0 leads to loss of two of the ^2H). The fraction of each fatty acid which was deuterated was calculated from peak areas for the M^+ and $(M + 4)^+$ or $(M + 2)^+$ ions for each fatty acid methyl ester. Correction was made for the fraction of $(M + 2)^+$ ions present due to normal isotope abundance in fatty acid standards (Alltech, Deerfield, IL). A similar procedure was used for 16:0 deuterated at other carbons.

Double-bond location in the monounsaturated fatty acids was determined from mass spectrometry of pyrrolidide derivatives prepared from fatty acid methyl esters (12).

RESULTS AND DISCUSSION

Supplementation of *Neurospora* cultures with 30 μM $18:1^{\Delta 9}$ or 16:0 had only a minor effect on the relative fatty acid composition of lipids, as shown in Figure 1A, B, and C. In previous studies (2–5), *Neurospora* lipids were enriched in the supplemented fatty acid, although these studies used higher concentrations of fatty acid than our study. We observed slightly increased concentrations of unsaturated fatty acids for $18:1^{\Delta 9}$-supplemented and of saturated fatty acids for 16:0-supplemented cultures, compared to unsupplemented cultures.

Over 97% of the exogenous [1-^{14}C]$18:1^{\Delta 9}$ was taken up by the cultures, and 59% of that taken up was converted to $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$ (Fig. 2A), and to a lesser extent (1–2% of total label), to $20:1^{\Delta 11}$, a minor fatty acid in *Neurospora*. Thus, the added $18:1^{\Delta 9}$ was readily desaturated and a small amount elongated. Specific activities (cpm/mmol) of the individual ^{14}C-labeled fatty acid differed (Fig. 2A); in phospholipid, which includes the phosphatidylcholine substrate for $18:1^{\Delta 9}$ and $18:2^{\Delta 9,12}$ desaturation, the specific activity of $18:2^{\Delta 9,12}$ was 0.8×10^9 cpm/mmol while the specific activities of $18:1^{\Delta 9}$ and $18:3^{\Delta 9,12,15}$ were greater (by 73 and 112%, respectively). This difference suggests preferential use of the [^{14}C]$18:2^{\Delta 9,12}$ for desaturation. The proportion of ^{14}C-label incorporated into each lipid class was similar to the proportion of its fatty acid mass, with the exception of the lower amount of label vs. mass in the glycolipid fraction (Table 1).

Cultures supplemented with [7,7,8,8-$^2H_4$]16:0 incorporated the deuterated fatty acid, with different levels of label incorporation in individual fatty acids (Fig. 2B). Twenty percent of the [^{2}H]16:0 was elongated to [^{2}H]18:0 as an end product, and both labeled compounds were incorporated into triacylglycerol. Further elongation of 18:0 to longer-chain saturated fatty acid (20:0, 22:0, and 24:0) in triacylglycerol and phospholipid was similarly efficient, with label in 20–22% of the longer-chain fatty acid. The relative proportion of [^{2}H]-labeled fatty acid in the lipid classes differed from the fatty acid mass (Table 1); a lower amount of label was incorporated into phospholipids, and a higher amount into triacylglycerol. This observation is consistent with the role of triacylglycerol as storage lipid that can accommodate a higher relative level of saturated fatty acid than phospholipids, which are desaturation substrates and, as components of membranes, appear to regulate fatty acid composition more strictly (9).

In contrast to the efficient incorporation of label from [^{2}H]16:0 into saturated fatty acids, label was relatively poorly incorporated into the major unsaturated fatty acids (Fig. 2B). Although the unsaturated fatty acids were present at 12 times the mass of 18:0 in the phospholipid and triacylglycerol, they contained only 37% of the incorporated [^{2}H]16:0. The lowest

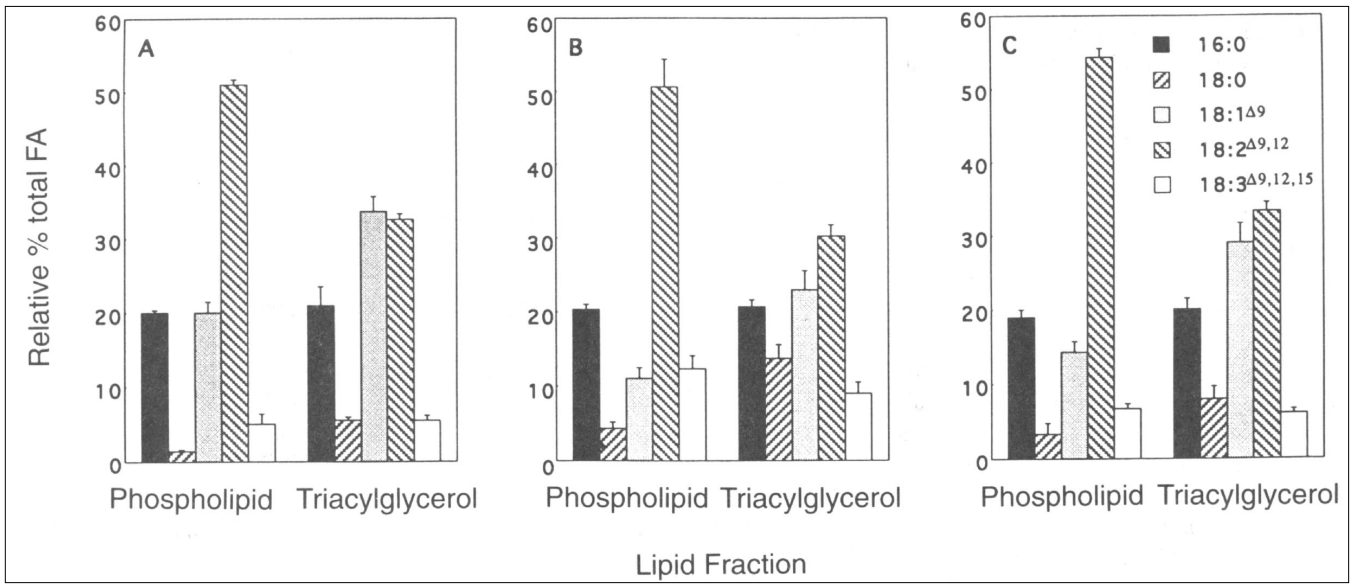

FIG. 1. Fatty acid (FA) composition of *Neurospora* lipids. A, 50 mL cultures supplemented with 30 μM [1-^{14}C]$18:1^{\Delta 9}$ (specific activity 3.3×10^9 cpm/mmol) as described in the Experimental Procedures section; B, cultures supplemented with 30 μM [7,7,8,8-^2H]$_4$16:0; C, unsupplemented cultures. Values are means ± SE of three or more experiments. (Balance in minor fatty acids contributes 2% or less of the total.)

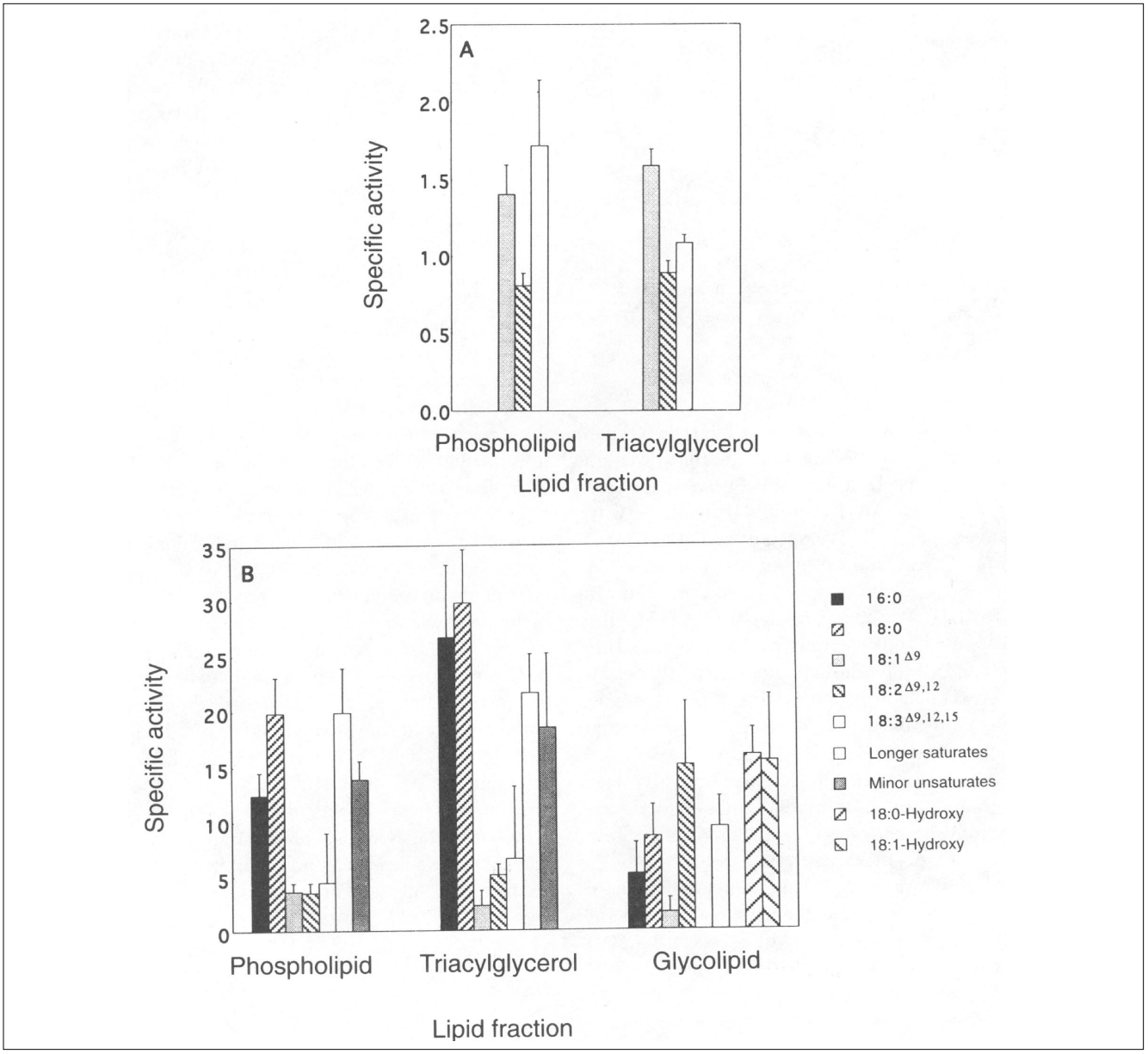

FIG. 2. Specific activity of fatty acids in *Neurospora* lipids. A, Cultures supplemented with 30 μM [1-^{14}C]18:1$^{\Delta 9}$ (label incorporation into glycolipids not detectable). Samples were analyzed for label incorporation by high-performance liquid chromatography and for fatty acid mass by gas chromatography with flame-ionization detection. Values are average specific activities (10^9 cpm/mmol) for six samples; error bars indicate SE calculated from normalized values. B, Cultures supplemented with 30 μM [7,7,8,8-$^2H_4$]16:0. Deuterium incorporation determined by gas chromatography/mass spectrometry with selective ion monitoring. Values are average specific activity (% deuterated) ± SE for three experiments. Long-chain saturated fatty acids, average for 20:0, 22:0, 24:0; minor unsaturated fatty acids, 16:1$^{\Delta 9}$ and 18:1$^{\Delta 11}$. Missing values (both panels) indicate that insufficient material was present to calculate specific activity.

level of deuteration was found in 18:1$^{\Delta 9}$. In phospholipid, the percentage deuterated 18:0 (specific activity, Fig. 2B) is five times greater than the percentage deuterated 18:1$^{\Delta 9}$. This five-fold dilution must occur as the result of endogenously produced 18:1$^{\Delta 9}$, indicating that desaturation of 18:0 to 18:1$^{\Delta 9}$ is more efficient for 18:0 synthesized *de novo* by the fatty acid synthase. The polyunsaturated fatty acids had higher levels of deuteration than 18:1$^{\Delta 9}$, indicating that once 18:1$^{\Delta 9}$ is formed, it is readily used by the desaturation pathway. The lower level of deuterated unsaturated fatty acids formed from the exogenous 16:0 is not due to inhibition of desaturation by [^{2}H]16:0, since the fatty acid composition of these cultures is similar to

TABLE 1
Relative Amounts of Total Fatty Acid in Lipid Fractions of *Neurospora* Cultures[a]

Supplement	Lipid fraction	Total fatty acid mass[b] (%)	Total label[b] (%)
[1-^{14}C]18:1$^{\Delta 9}$	Phospholipid	74.03 ± 4.20	71.97 ± 4.09
	Triacylglycerol	16.28 ± 3.67	18.98 ± 4.44
	Glycolipid	0.95 ± 0.30	0.26 ± 0.02
[7,7,8,8-$^{2}H_4$]16:0	Phospholipid	76.80 ± 5.89	58.81 ± 9.12
	Triacylglycerol	18.63 ± 1.03	35.07 ± 9.11
	Glycolipid	1.87 ± 0.34	3.15 ± 1.41

[a]Total fatty acid, [^{14}C]18:1$^{\Delta 9}$-supplemented, 911 µg/culture (average of 6); [^{2}H]16:0-supplemented, 1005 µg/culture (average of 3).
[b]Balance of material in remaining neutral lipids, including free fatty acid for 18:1$^{\Delta 9}$.

that of cultures grown without exogenous [^{2}H]16:0 (Figure 1B and 1C). A primary isotope effect that inhibits desaturation of only the deuterated 18:0 is possible, because the elongation product of [7,7,8,8-$^{2}H_4$]16:0 would be the [9,9,10,10-$^{2}H_4$]18:0. However, a similarly low specific activity of 18:1$^{\Delta 9}$ relative to its precursors and products was also observed when wild-type *Neurospora* cultures were supplemented with [2,2- or 16,16,16-^{2}H]16:0 (Table 2) or [1-^{14}C]16:0 (13). Since exogenous, deuterated 16:0 is efficiently elongated to 18:0, while the deuterated 18:0 produced is not efficiently desaturated to 18:1$^{\Delta 9}$, we conclude that elongation of the exogenous 16:0 to 18:0 occurs in a metabolic pool separate from that of *de novo* fatty acid synthesis.

[^{2}H]16:0 was also converted into palmitoleate (16:1$^{\Delta 9}$) and *cis*-vaccenate (18:1$^{\Delta 11}$) (Fig. 2B), products of a minor desaturation pathway in *Neurospora* (3). The 16:1$^{\Delta 9}$ results from formation of 16:0-CoA and desaturation, probably by the stearoyl-CoA Δ9-desaturase, which inserts a double bond in the 9,10-position of the fatty acid chain. The yeast and animal forms of this enzyme are capable of desaturating 16:0-CoA (14). Subsequent elongation of 16:1$^{\Delta 9}$ produces 18:1$^{\Delta 11}$.

Glycolipids are a relatively minor, though important, component of *Neurospora* lipids. Although 24-carbon saturated hydroxy fatty acids have been identified as the major components of *Neurospora* glycosphingolipids (15), we found that 60–70% of the total glycolipid fatty acid was C_{18} hydroxy fatty acid (2-hydroxystearate and 2-hydroxyoctadecenoate) and about 10% was C_{20}–C_{24} hydroxy fatty acid, with the remaining fatty acid not hydroxylated. Label from [^{2}H]16:0 was readily incorporated into both C_{18} hydroxy fatty acids (Fig. 2B). [^{2}H]16:0 incorporation into glycolipids was proportionally higher than [^{14}C]18:1$^{\Delta 9}$ incorporation (Table 1), presumably due to the 2-hydroxy fatty acid in this fraction being derived from elongation products of 16:0.

The percentage deuterated fatty acid (specific activity, Fig. 2B) present in saturated fatty acids ranged from 20–30%, and in minor fatty acids is 14–18%, while it ranged from 2–6% for the major unsaturated fatty acids. Thus, the exogenously added [^{2}H]16:0 was less efficiently utilized for the major desaturation pathway. We infer that the [^{2}H]18:0 produced by elongation of [^{2}H]16:0 is not as accessible to desaturation as 18:0 formed *de novo*. Therefore, 18:0 derived from exogenous [^{2}H]16:0 behaves as if in a separate metabolic pool. Our evidence is consistent with two pathways of biosynthetic reactions leading from 16:0. One is the main pathway leading to unsaturated fatty acids, originating from the fatty acid synthase reaction *via* elongation of 16:0 to 18:0, then desaturation to 18:1$^{\Delta 9}$. The other pathway leads to accumulation of saturated fatty acids. These appear to be formed primarily after thioesterase hydrolysis of the 16:0 fatty acid synthase product to free fatty acid which can be elongated to 18:0 and longer saturated fatty acids. While a small amount of this free fatty acid can reenter the pathway to 18:1$^{\Delta 9}$, much of it remains in the saturated fatty acid pathway and is directed as such into both storage and structural lipid.

Our results suggest a hitherto unrecognized control point that governs fatty acid composition. A mutation at this point could alter synthesis of saturated or unsaturated fatty acids. *Neurospora ufa* and *pfa* mutants (3–6), which are impaired in production of unsaturated fatty acids, have until now been presumed to have defects in desaturases, associated reductases, or in the synthesis of substrate lipids. These results, then, provide a new focal point for efforts in determining the regulation of fatty acid composition.

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TABLE 2
[^{2}H]-Fatty Acid Content of Each Fatty Acid Percentage[a]

Label position	16:0	18:0	18:1$^{\Delta 9}$	18:1$^{\Delta 9,12}$	18:1$^{\Delta 9,12,15}$	Long-chain	Minor unsaturates
[2,2-$^{2}H_2$]16:0	6.2	5.0	1.6	1.9	2.6	5.2	4.9
[16,16,16-$^{2}H_3$]16:0	6.9	4.6	No [^{2}H] detected	1.4	1.2	6.6	7.0

[a]Percentages of deuterated fatty acid for each fatty acid component of the total lipid, from 50 mL cultures grown with 30 µM palmitate deuterated in the 2,2- or 16,16,16-positions; results presented are the average of two determinations.

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Correlation of ATP/Citrate Lyase Activity with Lipid Accumulation in Developing Seeds of *Brassica napus* L.

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ABSTRACT: The temporal distribution of ATP/citrate lyase (ACL) activity in developing seeds of *Brassica napus* L. closely paralleled both that of acetyl-CoA carboxylase and the overall rate of lipid biosynthesis. Maximum ACL activities (250 nmol acetyl-CoA formed $min^{-1} \cdot$ g fresh seed) were recorded between 35 to 42 d after pollination and, if the *in vitro* data could be extrapolated to the situation *in vivo,* could account for half of the acetyl-CoA required for the measured rate of fatty acid biosynthesis during seed development. The enzyme appeared to be localized in a subcellular compartment, which was clearly separated from mitochondria on a sucrose gradient and by differential centrifugation, and which corresponded to the chloroplast organelle.

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During oilseed development, significant amounts of sugar provided by photosynthesis are converted to storage triacylglycerols by the developing seed. The pathway from sugars to storage oil has been extensively investigated in plants, and the main site of biosynthesis of fatty acids up to oleic acid occurs in the plastid stroma (1). This is in contrast to other eukaryotic cells, including yeasts, where fatty acid biosynthesis occurs in the cytoplasm. All plant fatty acids are synthesized ultimately from CO_2, but the point at which the pathway commits the fixed carbon to lipid biosynthesis is the conversion of acetyl-CoA to malonyl-CoA by the action of acetyl-CoA carboxylase (EC 6.4.1.2). However, as Ohlrogge and Browse (2) have elegantly summarized, "Our understanding of how carbon moves from photosynthesis into acetyl-CoA is clouded by an abundance of potential pathways. . . . It is more than likely that more than one pathway may contribute to maintaining the acetyl-CoA pool, and which pathway is used may vary with tissue, developmental stage, light/dark conditions and species."

The principal candidate route for acetyl-CoA formation is *via* pyruvate dehydrogenase (PDH) which acts on pyruvate transported from the cytosol into the plastid (3,4). There are, though, some objections to this as PDH activity is insufficient in nongreen plastids to account for observed rates of fatty acid biosynthesis (5). Roughan and Ohlrogge (6) have suggested that a route involving free acetate entering the plastids, there to be activated *via* acetyl-CoA synthetase, may be a key route. Malate (7) and glucose 6-phosphate (4) also have been proposed as precursors of acetyl-CoA either alone or in conjunction with other pathways. What is clear, however, is that the acetyl-CoA to be used in fatty acid biosynthesis must be synthesized in the subcellular compartment in which it is to be used since subcellular membranes are impermeable to acetyl-CoA (3). In plants, this means that acetyl-CoA for *de novo* fatty acid biosynthesis must be synthesized in the plastid.

Plants which produce oil-rich seeds accumulate large amounts (up to 60% in some seeds) of storage lipid and may therefore have methods of providing acetyl-CoA for this process which other nonoleaginous plants do not or are unlikely to have. The fact that seed tissues in oil-bearing plants synthesize so much oil and so rapidly in comparison to other plant tissues probably means that these tissues have a highly efficient, and probably highly seed-specific, system for synthesizing the metabolic precursors of fatty acids—including acetyl-CoA.

In eukaryotic cells, other than plants, fatty acid synthesis occurs in the cytoplasm. Citrate, produced *via* glycolysis and citrate synthase in the mitochondria, is exported into the cytoplasm and there cleaved by ATP/citrate lyase (ACL) (EC 4.1.3.8). The acetyl-CoA produced is used in fatty acid synthesis and the oxaloacetate recycled to the mitochondria. Not surprisingly, the activity of ACL is closely linked to the onset of lipogenesis in rats and humans (8–10) and also to the accumulation of lipid in oleaginous yeasts and fungi: the presence or absence of the enzyme either conferring or removing the capacity for oleagenicity from yeasts grown under the appropriate nutritional conditions (11–15).

There have previously been a number of reports of ACL activity in plant tissues. The activity of the enzyme has been linked to fatty acid biosynthesis (16–19), sesquiterpenoid biosynthesis (20), and carotenoid biosynthesis (21). We have therefore set out to investigate whether ACL is present in the developing seeds of *Brassica napus* L., as a typical oleagi-

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Abbreviations: ACC, acetyl-CoA carboxylase; ACL, ATP/citrate lyase; dpp, days postpollination; FW, fresh weight; G6PDH, glucose-6-phosphate dehydrogenase; ME, malic enzyme; PDH, pyruvate dehydrogenase.

nous plant, and to determine what role it may play in fatty acid biosynthesis.

MATERIALS AND METHODS

Preparation. Brassica napus L. cv. Weber, a rapid cycling variety, was grown in greenhouses, in soil-based compost. Plants were given a minimum of 16 h daylight, natural light being supplemented when necessary with artificial light. Flowers were hand-pollinated. Developing pods were harvested periodically up to 70 d after flowering and stored at 4°C until used. All seeds were used on the same day as they were harvested.

Crude extracts of developing *Brassica napus* L. seed were prepared by removing the seed from the pods and grinding 2 g (fresh weight) in a prechilled mortar with a prechilled pestle in 10 mL extraction buffer containing 100 mM KH_2PO_4, pH 7.2, 50 mM NaF, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM $MgCl_2$ and 1 mM *p*-aminobenzamidine as a protease inhibitor. The resulting homogenate was filtered through four layers of sterile muslin to remove large debris and then centrifuged at 36,000 × *g* for 30 min. The supernatant was removed, taking care not to disturb the lipid layer at the top of the tube, and this crude extract was used in the subsequent enzyme assays. ACL was assayed by the coupled spectrophotometric method (22) with reactions being routinely initiated with ATP after 5-min incubation. Fumarase (23), $NAD(P)^+$-malic enzyme (24), glucose-6-phosphate dehydrogenase (G6PDH) (25), and acetyl-CoA carboxylase (26) were assayed as indicated.

Density gradient centrifugation. Subcellular components were prepared at 4°C, on 12 mL step-gradients of between 30 and 60% (w/w) sucrose in equal 5% steps. Fresh tissue, 2 g, was roughly chopped in 5 mL 330 mM sorbitol, 100 mM KH_2PO_4, 0.1 mM EDTA, and 1 mM $MgCl_2$. The resulting fragments of seed were then pushed through a fine nylon mesh (mesh size approx. 0.1 mm), the pulp was collected, and 2 mL of the through-flow then loaded onto the top of the cold gradient. The gradients were unloaded from the bottom up in 1-mL fractions and particulate material pelleted by centrifugation. Protein was estimated by the Bradford method (27).

Differential centrifugation. Fresh seed tissue was collected about approximately 40 d postpollination (dpp) and homogenized as detailed in the Density gradient centrifugation section. After filtering the unfractionated homogenate through four layers of muslin, a crude plastid fraction was prepared by centrifugation (800 × *g,* 2 min at 4°C followed by 2000 × *g* for 10 min at 4°C). The supernatant was removed and retained and the pellet gently resuspended in 3 mL homogenization buffer (see above). This was termed the crude plastid fraction. The supernatant was then centrifuged at 12,000 × *g* for 10 min at 4°C and the supernatant removed. This was termed the crude cytosolic fraction. The 12,000 × *g* pellet was resuspended in 3 mL homogenization buffer and termed the crude mitchondrial fraction.

Radioactive incorporation. Incorporation experiments of [1-^{14}C]acetic acid and [1,5-^{14}C]citric acid into lipid were performed on crude plastid, crude mitochondrial, and crude cytosolic fractions. The plastid fraction was prepared by centrifugation of the homogenate (described above for the step gradients) at 800 × *g* for 2 min at 4°C followed by 2000 × *g* for 10 min at 4°C. The resulting supernatant was removed and retained and the pellet carefully resuspended in 3 mL homogenization buffer. To obtain the mitochondrial fraction, the retained supernatant was centrifuged at 12,000 × *g* for 10 min at 4°C and the pellet was resuspended in a 3 mL homogenization buffer. The supernatant from this centrifugation was termed the cytosolic fraction. Between 100 µL and 1 mL of each fraction were incubated in a reaction mixture containing, in 3 mL, $KHCO_3$ (30 µmol), $MgCl_2$ (5 µmol), β-mercaptoethanol (2 µmol), ATP (15 µmol), NADPH (2.5 µmol), NADH (2.5 µmol) and CoA (2.5 µmol) plus either [1,5-^{14}C]citric acid (10 nmol, 1 µCi) and trisodium citrate (1.2 µmol) or [1-^{14}C]acetic acid (18 nmol, 1 µCi) and sodium acetate (1.2 µmol). Reactions were incubated for 2 h at 30°C with constant, gentle shaking and illumination in small, stoppered Warburg manometer flasks with a center well containing a filter-paper wick soaked in 0.5 mL ethanolamine to absorb CO_2. After 2 h, the flasks were removed from the shaker, placed on ice in the dark, and the reactions stopped by adding 0.5 mL 15% (wt/vol) methanolic KOH. The wicks and ethanolamine were removed and the center wells washed with 1.5 mL polyethylene glycol monoethyl ether. The wicks and washings were pooled, placed in plastic scintillation vials with 10 mL scintillation fluid, and counted directly by liquid scintillation. The reaction mixture was transferred to a 50 mL centrifuge tube, and the inside of each manometry flask was washed with 2 mL distilled water and 2 mL chloroform/methanol (2:1, vol/vol). Washings and reaction mixture were combined and 5 mL chloroform/methanol (2:1, vol/vol) and 2 mL distilled water added. The aqueous phase was extracted three times with 5 mL chloroform/methanol (2:1, vol/vol) and the organic fractions combined and evaporated to dryness under a stream of N_2. The dried organic extract was dissolved in 10 mL scintillation fluid and counted. All radioactive counts were corrected for quenching using internal and external standards.

RESULTS

Enzyme assays for ACL were optimized using seeds 42 dpp. Maximum catalytic activity occurred at pH 7.8 with 50% of maximum activity at pH 6.8 and 8.4. Addition of citrate or glycerol to the extraction buffer had no stabilizing effect on the activity of the enzyme from *B. napus* L. The reaction was dependent on ATP and CoA and was optimal between 34 and 37°C.

Enzyme activities were measured during the course of seed development, from 14 dpp to final desiccation of the seeds which occurred approximately 70 dpp. Measurements of lipid content and enzyme activities are given in Figure 1. Results are presented in activities per mg fresh weight of the

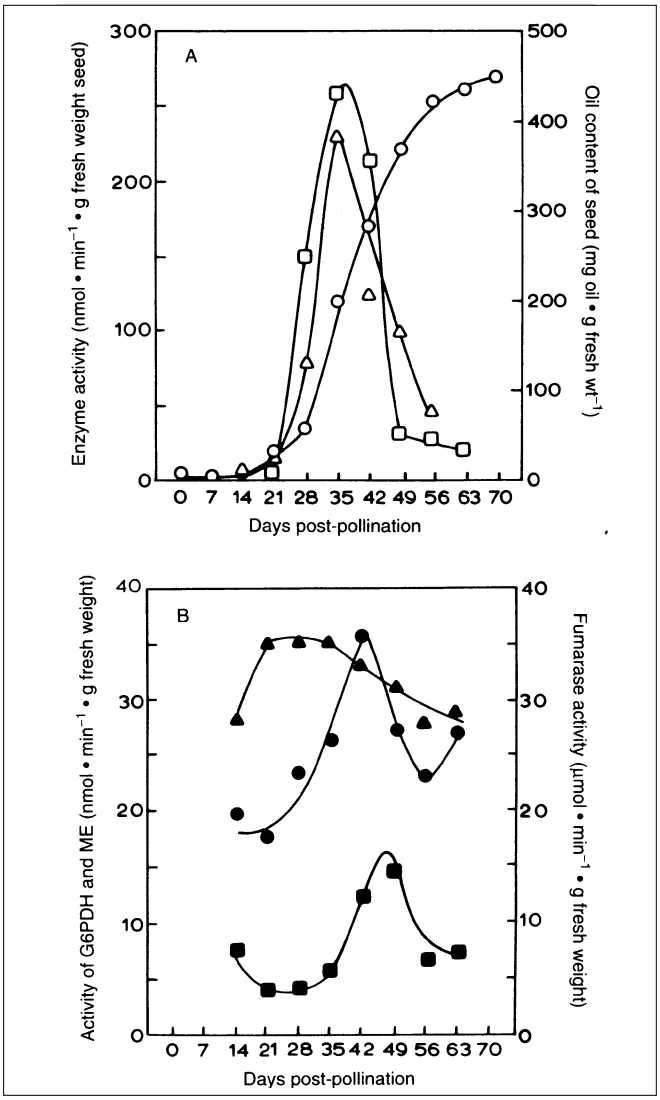

FIG. 1. Activities of various enzymes in developing, greenhouse-grown rape seed with the increase in oil content of the seeds. A: ATP/citrate lyase (△), acetyl-CoA carboxylase (□); total oil content of seeds (○); B: fumarase (▲), $NADP^+$-malic enzyme (ME) (■), and glucose-6-phosphate dehydrogenase (G6PDH) (●).

seeds to avoid errors associated with the accumulation of nonenzymatic, storage protein during seed development. The drawback of presenting the data in relation to seed dry weight is that during desiccation, seed weight inevitably drops and, as a result, the activity of the enzymes and lipid content may appear to rise. The results presented here indicated that this was occurring with unexpected increases in seed lipid between 63 and 70 dpp and in the activities of $NADP^+/NAD^+$-malic enzyme, glucose-6-phosphate dehydrogenase and fumarase between 56 and 63 dpp. ACL and acetyl-CoA carboxylase (ACC) activities, however, were not affected. Since this imprecision arose late in seed development and after the main phase of lipid accumulation had finished, it was considered acceptable to measure enzyme activities in terms of the fresh weight of seeds and particularly for determinations during early seed development.

ACL activity was first detected 14 dpp and reached a maximum of 232 nmol min^{-1} · g fresh weight (FW) at 35 dpp (Fig. 1A). It then decreased until it became undetectable at 70 dpp. The changes in the activity of the enzyme closely followed the increases and decreases in the rate of lipid accumulation in the seed. Changes in ACL activity also closely paralleled changes in the activity of ACC. Although ACC activity was slightly lower than ACL activity during the early part of seed development, the rate of increase of ACC activity was greater after 21 dpp and maximum ACC activity of 258 nmol min^{-1} · g FW was reached at the same time as maximum ACL activity. The activities of the other enzymes investigated did not show such a close correlation (Fig. 1B). Activity of NAD(P)-malic enzyme appeared to peak during the period of maximum oil biosynthesis although overall its activity was much lower than either that of ACL or ACC. The cytosolic and mitochondrial markers, respectively glucose-6-phosphate dehydrogenase (G6PDH) and fumarase, showed no correlation in activity during the period of maximum lipid accumulation; however the changes in fumarase activity were similar to those of malic enzyme, suggesting a possible, but functionally uncertain, linkage. Correlation coefficients between ACL and ACC activity and the rate of oil accumulation were 0.707 and 0.936, respectively, both of which are significant. Maximum catalytic activity of G6PDH at 261 nmol min^{-1} · g FW is sufficient to provide approximately 35% of the total NADPH required for the measured rate of lipid biosynthesis at this stage of seed development. This may explain why malic enzyme activities are so low since the extra NADPH which this enzyme produces, and is essential in animals and yeast, may not be needed as sufficient will be produced by the two enzymes of the pentose phosphate pathway, G6PDH and 6-phosphogluconate dehydrogenase, and by photosynthesis.

The fractions taken from the density gradients of homogenized rapeseed taken after 35 to 42 dpp were analyzed for ACL and fumarase activity, as well as for protein and chlorophyll concentration (Table 1). ACL activity was detected in fractions 10 to 2 from the density gradients with maximum activity in fractions 10, 8, and fraction 3. The fractions 10, 9, and 8 represent the cytosolic and broken organelle component of the homogenate while fractions 3 and 4 contain unbroken chloroplasts. As the chlorophyll concentration was higher in fraction 4 than in fraction 3, the latter possibly may be developing chloroplasts, with buoyant densities of between 1.2 and 1.22 g/mL. Fraction 6 gave the highest fumarase activity, indicating that this fraction contained unbroken mitochondria with a buoyant density of 1.14 g/mL. High fumarase activities in fractions 10 and 9 are indicative of considerable mitochondrial rupture during preparation of the homogenates and the gradients: 73% of the total fumarase activity was found in fractions 10 to 8. Equivalent levels of plastid breakage could be assumed suggesting that the ACL activity in fractions 10 to 8 was the result of the plastid rupture and not native, cytoplasmic ACL.

To establish whether ACL was isolated within the plastid stroma or was membrane-bound, the pellet recovered from

TABLE 1
Distribution of ATP/Citrate Lyase (ACL) and Fumarase Activities[a]

Fraction	Buoyant density (g mL^{-1})	Likely organelle	Protein (mg mL^{-1})	Chl (mg mL^{-1})	ACL (nmol · min^{-1} · mg protein)	Fumarase (nmol · min^{-1} · mg protein)
1	1.24		0.3	0	0	0
2	1.22		1.1	0.4	4.4	0
3	1.20	Chloroplast	1.4	0.6	25.8	0
4	1.18	Chloroplast	2.5	0.9	15.7	0
5	1.16		1.2	0.4	6.9	39
6	1.14	Mitochondrion	0.9	0.1	8.5	336
7	1.12		1.5	0.1	7.2	56
8	1.06	Cytosol	2.3	0.5	23.3	213
9	1.06	and broken	1.9	0.1	18.1	282
10	1.06	organelles	1.8	0.1	22.6	246

[a]Along with protein and chlorophyll contents, in homogenates of developing rapeseed (taken 35 to 42 dpp) and fractionated by centrifuging in a 30 to 60% (wt/vol) stepped sucrose density gradient; Chl, chlorophyll; dpp, days postpollination.

fraction 3 of the density gradients (see Table 1) was resuspended in homogenization buffer with and without sorbitol and recentrifuged (40,000 × *g* for 1 h). ACL activity remained in the soluble (i.e., nonsedimentable) fraction in both cases indicating that it was not a membrane-associated activity in the original fraction 3. ACL therefore appeared to be genuinely associated with the plastid fraction.

In an attempt to establish a clear role for ACL within developing rapeseed, further preparations of plastids, mitochondria, and cytosol were obtained by differential centrifugation. Each preparation was measured for ACL and fumarase activity to establish the degree of contamination of each fraction with the other. The results (Fig. 2) indicated that organelles had been successfully separated. The total ACL activity recovered in the plastid fraction was over 70% of the total ACL activity in all three fractions. Each of these fractions was then tested for its ability to convert either citrate or acetate into lipid and CO_2 (Fig. 3). In crude preparations of plastids, acetate was clearly a better precursor of fatty acid biosynthesis than was citrate. This has already been widely reported (1,2,28). However, the cytosolic fractions also efficiently incorporated label from both acetate and citrate into lipids with citrate being the marginally more efficient precursor. Mitochondrial fractions did not efficiently incorporate label from either acetate or citrate into lipid but were extremely effective at converting both precursors to CO_2.

DISCUSSION

Although the availability of acetyl-CoA is probably not limiting the rate of fatty acid biosynthesis in developing rapeseed under physiological conditions, it is obvious that an adequate supply of this precursor is needed to initiate and maintain the process. As indicated at the beginning of this paper, the origins of acetyl-CoA for fatty acid biosynthesis in plant tissues still appear to be uncertain (see Refs. 2 and 4 for further details) but, given the large difference in quantities of acetyl-CoA needed in developing rapeseed in comparison to leaf tissue, it would not be surprising for an additional and specific pathway to be operating in these particular tissues. The high activity of ACL, its close relationship to the rate of oil biosynthesis, and the localization of the enzyme within the plastid would all suggest that a significant portion of the required acetyl-CoA was being provided *via* this enzyme in the tissues studied here. Peak *in vitro* ACL activities reported (232 nmol min^{-1} · g FW; see Fig. 1A) would produce, if they occurred

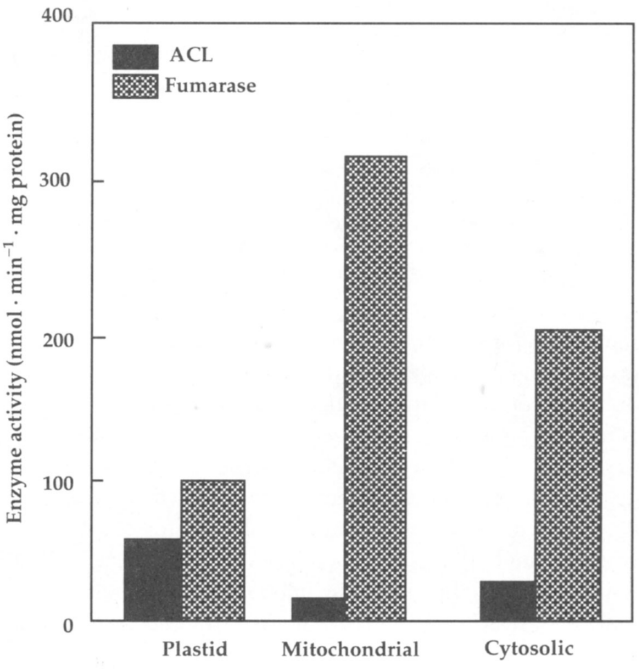

FIG. 2. Activities of ATP/citrate lyase (ACL) and fumarase in plastid, mitochondrial and cytosolic fractions of seed tissue prepared by differential centrifugation (see the Materials and Methods section). The total amount of protein in each of the three fractions was, respectively, 4, 1, and 2.5 mg.

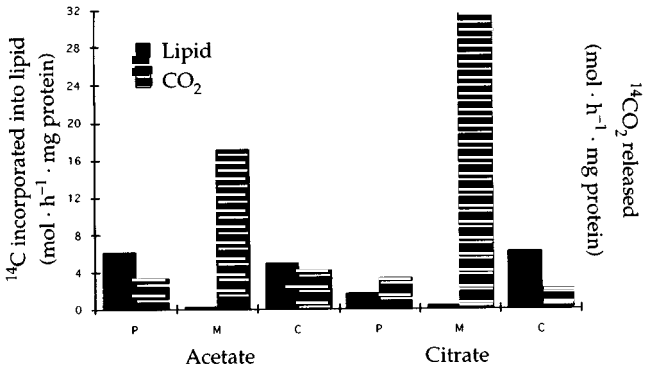

FIG. 3. Incorporation over 2 h of [1-^{14}C]acetate (1.22 μmol, 1 μCi) or [1,5-^{14}C]citrate (1.21 μmol, 1 μCi) into lipid (chloroform/methanol soluble fraction) or released as $^{14}CO_2$ by isolated subcellular fractions of developing rapeseed prepared as in Figure 2. P, plastid; M, mitochondrial; C, cytosolic fractions. No allowance is made for citrate only providing 1 mol acetate for fatty acid biosynthesis: i.e., effectively only 50% of the radioactivity of the acetate is available for incorporation into lipid.

in vivo, sufficient acetyl-CoA for the synthesis of 10.6 mg palmitate per day. This compares to a rate of lipid accumulation at this stage of seed development of just under 20 mg per day per g FW. On the basis of this, ACL could be providing up to half of the acetyl-CoA needed for fatty acid biosynthesis in these oleaginous seeds at this stage of their development and perhaps even a higher proportion if the assay of ACL activity was suboptimal.

The results from the density gradients appear to suggest that ACL is present in a plastid with a buoyant density 1.20 to 1.22 g/mL. As the same fractions contain the second highest chlorophyll concentrations of any of the fractions, this would suggest that the organelles are chloroplasts. This conclusion is consistent with previous reports of ACL in germinating castor bean endosperm which indicated that the enzyme was plastid localized (16). The confirmed presence of ACL in a plastid further supports the proposition that this acetyl-CoA is for fatty acid biosynthesis. If this is the case, it would indicate that at least one of the pathways to fatty acid biosynthesis in rapeseed may be comparable to the pathway found in mammals and oleaginous microorganisms (12–15).

Although some lipid synthesis was found in the cytosolic fraction (Fig. 3), this may have been as a result of the release of plastidic components. However, the incorporation of citrate into lipid by the cytosolic fraction may have a number of explanations: possibly, and some results from the density gradients support this proposition, native ACL is present in the cytoplasm, and the incorporation is a result of citrate cleavage to acetyl-CoA and its subsequent use in fatty acid chain elongation. Alternatively, the ACL found in the cytosolic fraction may be a result of plastid breakage, releasing both ACL and other components of the fatty acid synthesis machinery.

The results presented here demonstrate that ACL activity is present in plant tissues and is at its highest activity when the physiological demand for acetyl-CoA is highest. We therefore suggest that the role of this enzyme in plants is to maintain the cellular flux of the acetyl-CoA during those periods when lipid is being synthesized as a storage material in seeds. ACL activity varies in response to demand for acetyl-CoA and therefore would only be expressed at strategic times during plant development. Acetyl-CoA carboxylase, which must work in conjunction with ACL, parallels the activity of ACL, suggesting that a coordinated response is occurring during seed development. ACL would not be part of the "housekeeping" system but may be activated when the need is greatest. It would be less complicated for the plant to maintain a number of enzymes at fairly constant activities, and respond to increases and decreases in demand by activating or inhibiting one enzyme, than to keep constant and fine control of a large number of different enzymes. If this is the case, ACL activity should be detectable in plants under conditions where there is a large demand for acetyl-CoA for biosynthetic processes irrespective of what these might be.

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Digestion of the 1-*O*-Alkyl Diacylglycerol Ethers of Atlantic Dogfish Liver Oils by Atlantic Salmon *Salmo salar*[1]

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ABSTRACT: Dogfish *(Squalus acanthias)* liver poses a waste disposal problem in Canada because it is not utilized for any commercial purpose. The liver of Atlantic dogfish, which is often up to 20% of the weight of the fish, contains 40–70% oil. The oil contains about 30–40% 1-*O*-alkyl diacylglycerol ethers (DAGE) which render it unacceptable for human use, and it has also not been considered satisfactory for animal feed use. Polyunsaturated fatty acids (20:5n-3 and 22:6n-3) are present in dogfish liver oils at levels comparable to those in herring oil. Dogfish liver oil could be a source of essential fatty acids for Atlantic salmon *(Salmo salar),* but their ability to hydrolyze DAGE from dogfish oil has not been examined. Experiments were designed to measure the digestibility of fatty acids of DAGE in salmon. The fatty acid moieties were liberated by the digestive enzymes of the fish and made readily available as a source of energy. The 1-*O*-alkylglycerol ether moiety was absorbed to a small extent but should not constitute a health problem in either the fish or the human fish consumer. The long-chain polyunsaturated fatty acids were particulary well absorbed, with an apparent digestibility in salmon of 87–95% when feeding on dogfish liver oil. The total fatty acids and other lipids were in fact both absorbed to the extent of approximately 85%.
Lipids 32, 19–30 (1997).

The Atlantic dogfish *Squalus acanthias* is a very large resource (1,2). A growing fishery in southwestern Nova Scotia sells backs, bellies, skins, and fins to different markets. There is, however, a waste disposal problem with the livers, which amount to 20% of the weight of the fish and are at least 40% oil. This oil is easily recovered (3,4) and contains approximately 30% 1-*O*-alkyl diacylglycerols (DAGE), usually simply called glyceryl ethers, rendering it unfit for human use as the 1-*O*-alkylglycerol ether proper cannot be absorbed, and similarly it is not acceptable for most animal feed purposes (chicken, pigs, etc.) (5). The popular use of glyceryl ethers (DAGE) as a descriptive term should be restricted to 1-*O*-alkylglycerol ethers. The balance of the oil is triacylglycerol (TAG) with 1–5% free fatty acids (FFA). The FFA are produced by enzymatic hydrolysis, their proportion reflecting storage and handling. Marine oils are sources of the longer-chain polyunsaturated fatty acids of the n-3 (ω-3) family, especially eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). These are essential for the diets of Atlantic salmon (6,7), and we wished to examine their availability from DAGE *in vivo*. The DAGE are widely distributed in nature, and their biological role is highly significant (8). It is common knowledge that GE are found in marine animals of various types (8–11). Lie and Lambertsen (12) examined the digestion of these lipids in Atlantic cod (*Gadus morhua*). Other predatory fish such as Atlantic salmon would presumably be able to digest the DAGE abundant in marine animal prey.

Determination of lipid absorption *in vivo* requires determination of lipid in the food and unabsorbed residues in feces. This is generally achieved either by collection of total fecal matter excreted after consumption of a known amount of food, or measurement of the concentration of a marker in both feed and feces. Total collection of fecal matter from fish is tedious and often impossible. Chromium oxide is the most commonly used digestibility marker (13,14); however, it has two main disadvantages as a lipid digestibility marker in that it is not fat-soluble and has to be determined separately from fat or fatty acids. Improved technology for studying the digestive process of fats in trout and salmon has recently been developed by Sigurgisladottir *et al*. (15) employing cholestane as a digestibility marker. The present study, designed to evaluate the ability of Atlantic salmon to hydrolyze DAGE of Atlantic dogfish liver oil and to determine the digestibility

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Abbreviations: DAGE, 1-*O*-alkyl-2,3-diacylglycerol ether; DAG, diacylglycerol; GE, 1-*O*-alkylglycerol ether; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acid; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectroscopy; MAG, monoacylglycerol; TAG, triacylglycerol; TLC, thin-layer chromatography; TLC/FID, thin-layer chromatography with flame ionization detection.

of all the fatty acids in dogfish liver oil by Atlantic salmon smolts, was based on the use of cholestane as an indicator of fat absorption.

MATERIALS AND METHODS

Fish and diets. Sixty Atlantic salmon averaging 500 g each were acclimated to feed, aquaria, and standardized environmental conditions at the Department of Fisheries and Oceans, Halifax. Six tanks were used, each containing 10 Atlantic salmon smolts, allowing duplication of three dietary groups. Fresh water was supplied to each tank at a flow rate of 3 L/min and maintained at a temperature of 17 ± 1°C. The photoperiod was controlled automatically (scheduled as 12 h of light and 12 h of dark). Feeding to satiation was by hand twice during the light period.

A casein–gelatin based purified diet which contained 30% water was used in this study. Three diets were formulated (Table 1), with the diets differing only in two lipid sources or these in a 1:1 ratio (15% of diet). The following lipid supplements were tested: 15% Atlantic dogfish liver oil (diet 1), a mixture (1:1) of Atlantic dogfish liver oil and herring oil (diet 2) or 15% Atlantic herring oil (diet 3). The dogfish liver oil used in this experiment was extracted from commercial Nova Scotian dogfish livers by a conventional heating method (3), and commercial herring oil was obtained from Department of Fisheries and Oceans stocks. Cholestane (Sigma, St. Louis, MO) was dissolved in the various lipid sources, before they were mixed with the basal diet, to give a level of 0.7 mg/g diet.

Diet, fecal, and muscle lipid analyses. Duplicate samples of each experimental diet (10 g), sampled 15 d apart, were weighed, placed in a beaker with 50 mL of water and sonicated for 20 min (16). The solution was then mixed in a Waring Blendor® for chloroform/methanol extraction of lipid by the method of Bligh and Dyer (17). After two weeks on the experimental diets fed *ad lib*, the fish in each tank were anaesthetized by immersion in fresh water containing MS222 (ethyl 3-aminobenzoate methanesulfonate); feces were then collected from the rectal region by applying gentle pressure to the surface of the fish. The fish were returned to the tank and the procedure was repeated after five days. Samples from duplicate tanks were pooled on each occasion. A small proportion of each feces sample was dried at 105°C to constant weight for moisture analyses. The remaining part of the sample (four for each diet) was used for lipid extraction. The recovered fatty acids (as methyl esters) and the cholestane were determined in duplicate by capillary gas–liquid chromatography (GLC). The cholestane in diets and feces was applied to lipid recovery by modifying the calculations for the Cr_2O_3 method of Arthur (18).

Fish were killed by a blow on the head and filleted. Muscle from fish in each duplicate tank was pooled and extracted separately by the method of Bligh and Dyer (17). Each lot was then analyzed for lipid classes.

Isolation of chylomicrons. On two occasions all fish from each tank were sampled six hours after feeding. A blood sample was drawn from the experimental fish in a heparinized syringe, and after coagulation, blood serum was separated by low-speed centrifugation (3,000 × *g*) for 20 min.

All serum samples from each tank were pooled for analysis of chylomicrons. An antibacterial agent (0.01% sodium azide) and EDTA (1 mg/mL) were added in order to protect the chylomicrons from any oxidative degradation which might be catalyzed by free metal cations. Chylomicrons were isolated from serum by flotation after centrifugation at 15,000 × *g* for 30 min. Fractionations were performed at 10°C in a Beckman L5 50B centrifuge with a 50 Ti rotor (Fullerton, CA) according to Fremont *et al*. (19). Lipid class analyses were conducted in duplicate on each chylomicron sample.

Lipids were extracted from feces, muscle, and chylomicrons by the method of Folch *et al*. (20) as modified by Christie (21). Moistures of diet and feces were determined using recommended procedures (22). Individual lipid mixtures, isolated lipids, methyl esters, etc. were always analyzed in duplicate, as a minimum, but if two samples were available the results were also averaged ($n = 4$) for standard deviations.

Lipid class determination and isolation. A Mark III Iatroscan thin-layer chromatography/flame ionization detection (TLC/FID) analyzer (Iatron Laboratories, Inc., Tokyo, Japan; Canadian Distributor, Scientific Products & Equipment, Concord, ON) was utilized for the quantitative analysis of the lipid classes from each sample after separation on silica-gel coated Chromarods-SIII (23). The general procedure was modified for partial scan and redevelopment as shown by Parrish (24). One to 5 μL of a chloroform solution of the sample was applied to the Chromarods with Drummond disposable micropipets. After spotting, rods were placed in a constant

TABLE 1
Composition of the Experimental Diet

Feed ingredients	Amounts (w/w%)
Casein	40.0
Gelatin	8.0
Dextrin	9.5
Starch	9.8
d-Glucose	4.0
Carboxymethylcellulose	2.0
Alpha cellulose	5.0
Vitamin mixture[a]	1.0
Mineral mixture[b]	4.0
DL-Methionine	0.5
L-Arginine-HCl	1.0
Choline chloride	0.2
Lipid supplement[c]	15.0
Cholestane[d]	0.07

[a]Vitamins added to supply the following levels (mg/kg unless otherwise stated); thiamine, 50; riboflavin, 60; d-calcium pantothenate, 200; biotin, 1; folic acid, 20; vitamin B_{12}, 0.1; niacin, 250; pyridoxine, 40; ascorbic acid, 1000; inositol, 400; vitamin A, 8000 IU; vitamin D, 2400 IU; vitamin E, 300 IU, and vitamin K 40 IU, respectively.
[b]Minerals added to supply the following levels (mg/kg): manganese, 50; iron, 60; zinc, 120; copper, 25.
[c]Lipid supplements used were as follows: diet 1, dogfish liver oil; diet 2, dogfish liver oil + herring oil (1:1 w/w); diet 3, herring oil.
[d]Cholestane was dissolved in the lipid supplements, prior to mixing.

humidity tank over a saturated sodium chloride solution for 10 min and then transferred immediately to the developing tank. The Chromarods-SIII were developed in a sequence of solvent systems to enhance separation of different lipid classes. Firstly, total lipid on the Chromarod-SIII was developed with hexane/diethyl ether/formic acid (97:3:1, by vol) for 55 min, to separate neutral from polar lipids; secondly, hexane/chloroform/isopropanol/formic acid (85:14.5:0.75:0.1, by vol) for 40 min served to partially separate DAGE from FFA; and thirdly, acetone 100% for 30 min was used to separate the GE, diacylglycerols (DAG), and monoacylglycerols (MAG) from polar lipids. Developed rods were placed in an oven at 110°C for 3 min to evaporate the solvents and then transferred to the scanning frame of the Iatroscan analyzer for each partial or complete scan.

Separate fractions of DAGE, TAG, and FFA were obtained from each lipid lot by preparative TLC by streaking on "Prekotes" (Adsorbosil-5 silica gel TLC plates, 20 × 20 cm; Applied Science Laboratories, College Park, PA). The solvent development system was hexane/diethyl ether/acetic acid (85:15:1, by vol). Bands were visualized by spraying the plate with a 0.01% solution of 2′,7′-dichlorofluorescein in ethanol and viewing under ultraviolet (UV) light. Each band was scraped off the TLC plate, and the silica gel extracted with a mixture of chloroform/methanol (50:50, vol/vol). The MAG plus free GE bands were pooled and rechromatographed to permit recovery of enough GE for one analysis of fecal material from diets 1 and 2. The isolated DAGE were saponified with KOH in ethanol according to AOCS Official Method Ca 66-53. The unsaponifiable matter containing 1-*O*-alkylglycerol ether was recovered with freshly distilled diethyl ether. The DAGE of muscle was estimated from the GE in the unsaponifiable matter from direct saponification of muscle total lipids because the amount of actual DAGE in muscle was too low for an effective TLC separation.

Preparation of derivatives of glyceryl ethers and hydrogenation. Isopropylidene derivatives of 1-*O*-alkylglycerols were prepared by acetonization at room temperature in the presence of perchloric acid according to a modification of the method of Wood (25). Briefly, 1 mL of acetone containing 0.1 μL of 70% perchloric acid was added to the sample. The mixture was allowed to stand at room temperature for 20 min and 1–2 drops of concentrated NH_4OH were added, then the reaction was terminated by adding water and the product was extracted three times with hexane/diethyl ether (50:50, vol/vol). Further purification of these derivatives was achieved by TLC using the solvent system hexane/diethyl ether/acetic acid (40:60:1, by vol) prior to analysis by GLC.

For hydrogenation, the lipid samples (10–20 mg) were dissolved in methanol containing sufficient (5–10 mg) platinum oxide catalyst and stirred under a current of hydrogen at atmospheric pressure and room temperature for 2 h (26). In all cases this achieved complete hydrogenation.

Preparation of methyl esters. Up to 20 mg of lipid, 1.0 mL hexane, and 1.0 mL of 8% BF_3–methanol solution were placed in a screw-capped (Teflon-lined cap) centrifuge tube. The tube was flushed with nitrogen, sealed, and heated for one hour at 100°C. After cooling, 3 mL of water was added to the tube and the hexane layer removed to another tube. The aqueous layer was again extracted two times with hexane (2 mL each). The combined hexane extract was washed once with 3 mL H_2O, dried over anhydrous Na_2SO_4, and concentrated as required for GLC analysis of the methyl esters.

GLC and GLC–mass spectrometry (MS) analysis. The alkyl chain compositions of the 1-*O*-alkyl-2,3-isopropylidene glycerol derivatives were determined by GLC using a Perkin-Elmer 8420 gas chromatograph (Norwalk, CT) fitted with a FID and a flexible fused-silica Omegawax-320 (bonded polyglycol phase) open-tubular column (30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA). The injector and detector were held at 250 and 270°C and the column at 210°C. The split ratio was 1:50. Helium was used as the carrier gas at a constant inlet pressure of 12 psig. Fatty acid compositions of the methyl esters of fatty acids were determined by GLC using the same instrument and column but with the following program of column temperature: initially 185°C for 8 min, then an increase in temperature of 3° per min to 230°C, and a final hold for 10 min. Helium was also used as the carrier gas at a pressure of 12 psig. The injection port and detector were maintained at 250 and 270°C, respectively, for all analyses. Only component fatty acids of biochemical or nutritional importance (27) are tabulated, but all GLC peaks were identified using authentic fatty acid methyl esters or other technology (21) and were converted to weight percentage by correcting for the FID response of different chainlengths (28).

For 1-*O*-alkylglycerol ether peak identification GLC–MS data were obtained using an ITD (Finnigan MAT 700 ion trap detector; San Jose, CA) operated with an IBM personal computer. The column was operated in a Perkin Elmer 990 gas chromatograph and passed through a heated transfer line to connect directly to the ITD. The column and isothermal conditions for the GLC part were the same as for the GLC–FID analysis of the isopropylidene derivatives.

RESULTS AND DISCUSSION

Atlantic salmon (*Salmo salar*) (29) and some other fish species, such as red sea bream, rockfish and globefish (30), have a limited capacity for chain elongation and desaturation of 18:3n-3 to 20:5n-3 and 22:6n-3 (7). These species therefore require a certain level of 20:5n-3 and 22:6n-3 in the diet. Although n-3 fatty acids are essential nutrients in salmonids generally (31), there might be an upper limit to the level at which n-3 fatty acids will promote healthy growth in salmonids [2.5% of the diet and higher (32)]. We (6,33) and others (34) have found that several types of fats are readily absorbed (15) by Atlantic salmon, but these have been practical diets including fish meal, etc. In this experiment the experimental period in our study of three weeks was too short to measure growth, but all fish accepted the semisynthetic diets and ate well except for a few hours after being handled.

Total fatty acids. The total fatty acids of the three dietary

TABLE 2
Principal and Biochemically Interesting Total Fatty Acids (w/w%)[a] in Diets[b] Fed to Atlantic Salmon and in Feces Recovered from Atlantic Salmon

	Diet fed			Feces		
Fatty acid	Diet 1	Diet 2	Diet 3	Diet 1	Diet 2	Diet 3
14:0	3.72 ± 0.25	6.01 ± 0.25	7.47 ± 0.25	3.13 ± 0.75	5.57 ± 0.55	7.07 ± 0.46
16:0	13.34 ± 0.55	12.27 ± 2.75	11.00 ± 1.40	14.97 ± 2.49	15.31 ± 4.44	11.70 ± 2.85
18:0	2.16 ± 0.05	1.43 ± 0.03	0.90 ± 0.30	2.77 ± 0.20	2.50 ± 0.70	1.38 ± 0.12
Total saturated	21.0	21.6	21.1	22.9	25.3	21.8
16:1n-7	5.26 ± 0.25	6.05 ± 0.16	6.40 ± 0.25	3.49 ± 0.96	3.90 ± 0.81	4.23 ± 1.47
18:1n-9	11.95 ± 0.81	9.24 ± 0.95	6.92 ± 0.10	8.75 ± 2.47	6.74 ± 1.64	6.15 ± 1.91
18:1n-7	1.22 ± 0.05	2.18 ± 0.25	1.75 ± 0.10	2.50 ± 0.31	2.03 ± 0.19	1.77 ± 0.29
20:1n-(9+11)	14.46 ± 1.10	15.22 ± 1.95	16.54 ± 1.45	16.89 ± 1.55	16.26 ± 1.52	18.42 ± 2.85
20:1n-7	2.69 ± 0.15	0.82 ± 0.02	0.49 ± 0.01	1.54 ± 0.05	0.94 ± 0.05	0.57 ± 0.05
22:1n-(11+13)	16.86 ± 1.40	20.24 ± 2.69	24.17 ± 0.75	23.70 ± 3.39	27.13 ± 6.80	30.91 ± 7.05
22:1n-9	4.13 ± 0.03	2.51 ± 0.01	1.24 ± 0.05	5.48 ± 1.04	3.27 ± 0.87	1.46 ± 0.18
Total monounsaturated	59.3	58.9	60.3	66.3	63.6	66.9
18:2n-6	1.97 ± 0.16	2.01 ± 0.95	1.85 ± 0.07	0.99 ± 0.28	0.90 ± 0.16	0.91 ± 0.31
18:3n-3	0.72 ± 0.01	0.80 ± 0.03	0.82 ± 0.03	0.41 ± 0.12	0.42 ± 0.08	0.41 ± 0.08
18:4n-3	1.07 ± 0.05	1.62 ± 0.03	2.02 ± 0.02	0.31 ± 0.08	0.51 ± 0.16	0.66 ± 0.25
20:4n-6	0.26 ± 0.05	0.21 ± 0.02	0.18 ± 0.05	0.15 ± 0.07	0.11 ± 0.09	0.15 ± 0.05
20:5n-3	3.86 ± 0.25	4.35 ± 0.15	4.86 ± 0.35	0.87 ± 0.33	1.34 ± 0.29	1.72 ± 0.52
22:5n-6	0.29 ± 0.02	0.20 ± 0.01	0.13 ± 0.05	0.29 ± 0.03	0.39 ± 0.18	0.31 ± 0.04
22:5n-3	1.37 ± 0.10	1.21 ± 0.70	0.83 ± 0.07	0.65 ± 0.11	0.83 ± 0.14	0.73 ± 0.01
22:6n-3	6.02 ± 0.40	4.72 ± 0.90	3.76 ± 0.11	3.95 ± 1.42	3.51 ± 0.90	3.42 ± 1.59
Total polyunsaturated	19.8	19.5	18.6	10.9	11.2	11.3

[a]Mean and standard deviation of two analyses for diets, four analyses for feces.
[b]Added fats were: diet 1, dogfish liver oil only; diet 2, dogfish liver oil and herring oils mixed 1:1; diet 3, herring oil only.

fats (Table 2) were not radically dissimilar in most respects since the herring and dogfish share the same habitat, and the former is probably a major food item for the latter. The herring oil had higher levels of 22:1 than of 20:1 fatty acids, often a characteristic of this oil (35). The dogfish liver oil details resembled that for contemporary dogfish liver oil produced in Great Britain (4). In a report on Pacific dogfish liver oil (36), the 20:1 and 22:1 were much lower, and 18:1 much higher, than in the Atlantic oils. Other fatty acids were similar in proportions. The Atlantic dogfish liver oil had less 14:0 and more 18:0 than the herring oil, and also more 18:1n-9 but less 18:1n-7. All of these differences fitted the composition of the equal amounts of the oils blended for diet 2. The total fatty acids of the feces (Table 2) showed the same trends in the three saturated fatty acids, the 18:1 fatty acid, and the 22:1 fatty acids. In the latter a curiosity was the higher proportion of 22:1n-9 relative to 22:1n-(11+13) in the diets from the dogfish liver oil relative to those from the herring oil diet (Table 2). This was faithfully reproduced in the fecal fatty acids (Table 2). The ready absorption of 18:4n-3 is indicated by the two-thirds decrease of this polyunsaturated fatty acid which was similar to the excellent absorption of 20:5n-3. The 22:6n-3, in contrast, was presumably less well hydrolyzed and/or absorbed, but in all three cases the relative proportions among the three diets were preserved in the fecal total fatty acids. Overall the similarity in the composition of dietary fatty acids and those of feces reflected unhydrolyzed TAG as well as other lipid classes, but primarily unabsorbed FFA (Table 3).

In a previous study (15) we have confirmed the work of others, especially of Lie and Lambertsen (12) for cod to the effect that shorter chains of fatty acids were favored for hydrolysis and absorption over longer chain lengths among the saturated and monounsaturated fatty acids. We also had found the absorption of 20:5n-3 and 22:6n-3 to be excellent (≥95%). In the present study the digestibility of the saturated fatty acids declined with chainlength as expected (Fig. 1). The monounsaturated fatty acids followed suit. In the latter group the principal isomers 16:1n-7 and 18:1n-9 were almost equally well absorbed, but there was less absorption of 20:1 and even less of 22:1n-(11+13). Although the percentage numbers for digestibility for these two types of fatty acids were slightly less than those found previously in our laboratory (15) for Atlantic salmon, those of both 20:5n-3 and 22:6n-3 were again nearly the same with that of 20:5n-3 slightly exceeding 22:6n-3 (Table 4, Fig. 1). This was also found in Atlantic cod by Lie and Lambertsen (12). The total fatty acids and lipids were, in fact, both absorbed to the extent of approximately 85% (Fig. 1D). This argues for a minimal effect of the DAGE found in the dogfish liver oil on the total digestive process in Atlantic salmon.

Fecal lipids. Figure 2 compares the lipids of the three diets with the fecal lipids when examined qualitatively by plate TLC. An unexpected finding was the presence of cholesteryl

TABLE 3
Lipid Class Composition (w/w%)[a] of Experimental Diets[b], Feces, Muscle and Chylomicron from Atlantic Salmon as Determined by TLC/FID

Component	Diet 1	Diet 2	Diet 3
Diet			
Free fatty acids	0.3 ± 0.02	1.0 ± 0.06	2.2 ± 0.06
Diacylglycerol ether	18.2 ± 1.37	10.4 ± 1.45	n.d.
Triacylglycerols	79.6 ± 1.37	86.4 ± 3.28	95.3 ± 2.42
Sterols	0.5 ± 0.01	0.8 ± 0.03	1.0 ± 0.31
Polar lipids	1.4 ± 0.05	1.4 ± 0.12	1.5 ± 0.08
Feces			
Steryl/wax esters	0.5 ± 0.18	0.8 ± 0.09	1.1 ± 0.46
Free fatty acids	57.6 ± 3.24	50.6 ± 4.05	42.9 ± 5.76
Diacylglycerol ether	trace	trace	n.d.
Triacylglycerols	8.3 ± 1.65	9.2 ± 2.10	12.9 ± 1.78
Sterols	4.9 ± 0.82	6.5 ± 1.01	7.3 ± 0.85
Diacylglycerols	0.7 ± 0.38	1.9 ± 0.09	2.3 ± 0.37
Monoacylglycerols +GE[c]	11.6 ± 1.84	15.1 ± 1.43	18.1 ± 2.33
Polar lipids	16.4 ± 3.48	15.9 ± 1.00	15.4 ± 0.28
Chylomicrons			
Steryl/wax esters	14.0 ± 2.90	9.8 ± 0.40	13.1 ± 2.07
Free fatty acids	trace	trace	trace
Diacylglycerol ether	14.2 ± 5.90	7.3 ± 2.18	n.d.
Triacylglycerols	31.1 ± 0.99	32.9 ± 1.40	30.6 ± 0.37
Sterols	10.2 ± 0.81	9.2 ± 0.77	11.4 ± 2.09
Polar lipids	28.0 ± 7.24	41.0 ± 3.56	45.0 ± 0.42
Muscles			
Free fatty acids	trace	trace	trace
Diacylglycerol ether	0.03 ± 0.00[d]	trace	n.d.
Triacylglycerols	92.07 ± 3.12	91.8 ± 1.84	91.1 ± 2.71
Sterols	0.5 ± 0.01	0.6 ± 0.02	0.5 ± 0.04
Polar lipids	7.3 ± 2.15	7.6 ± 0.42	8.4 ± 1.56

[a]Standard deviation based on four determinations, see text for details; n.d., not detected.
[b]Added fats were: diet 1, dogfish liver oil only; diet 2, dogfish liver oil and herring oil mixed (1:1, w/w); diet 3, herring oil only.
[c]Only diets 1 and 2 included 1-*O*-alkyl-2,3-diacylglycerol ether (DAGE).
[d]This value was obtained from recovery of unsaponifiables, separation of 1-*O*-alkylglycerol ether on thin-layer chromatography with weight recovery, and calculation of DAGE from fatty acid data.

TABLE 4
Apparent Digestibility (%)[a] of Fatty Acids by Atlantic Salmon Fed Different Diets[b]

Fatty acid	Diet 1	Diet 2	Diet 3
14:0	82.1 ± 3.21	79.3 ± 5.48	77.2 ± 5.63
15:0 iso	80.6 ± 2.96	80.9 ± 4.92	78.0 ± 5.21
15:0 anteiso	85.9 ± 1.85	85.1 ± 3.75	81.0 ± 4.19
15:0	77.9 ± 4.09	76.0 ± 6.19	74.7 ± 5.96
16:0	75.6 ± 3.83	74.3 ± 6.58	71.9 ± 6.93
18:0	72.2 ± 4.54	69.7 ± 7.20	64.4 ± 8.26
20:0	59.4 ± 7.03	59.7 ± 9.93	58.2 ± 6.05
16:1n-7	86.7 ± 7.31	87.4 ± 3.23	86.9 ± 3.23
18:1n-9	85.3 ± 6.81	85.5 ± 3.37	84.2 ± 3.91
18:1n-7	80.7 ± 5.73	79.3 ± 5.19	78.1 ± 5.49
20:1n-(9+11)	75.0 ± 5.09	71.8 ± 6.78	70.9 ± 7.09
20:1n-7	73.1 ± 5.41	70.1 ± 7.57	69.6 ± 7.88
22:1n-(11+13)	68.8 ± 4.76	62.2 ± 8.79	60.0 ± 9.87
22:1n-9	70.8 ± 4.57	60.0 ± 9.89	65.2 ± 9.30
22:1n-7	61.9 ± 7.28	68.5 ± 7.20	52.7 ± 5.67
18:2n-6	90.3 ± 4.17	90.8 ± 2.45	90.4 ± 0.65
18:2n-4	73.8 ± 13.49	69.7 ± 7.22	76.7 ± 5.87
18:3n-3	88.8 ± 5.27	94.7 ± 1.34	89.4 ± 2.84
18:4n-3	94.2 ± 2.52	94.7 ± 1.34	94.7 ± 1.30
20:4n-3	90.8 ± 1.50	91.7 ± 2.24	88.4 ± 2.89
20:5n-3	95.6 ± 2.49	94.1 ± 1.43	93.6 ± 1.56
22:5n-3	90.2 ± 3.41	80.0 ± 5.47	76.5 ± 6.83
22:6n-3	87.0 ± 7.19	86.7 ± 3.17	87.2 ± 3.39
Total fatty acids	86.4 ± 3.00	85.1 ± 1.83	84.8 ± 3.43
Lipids[c]	84.2 ± 1.98	85.8 ± 2.77	86.2 ± 2.37

[a]Data are the mean ± SD of four determinations of the proportion (%) of the fatty acids.
[b]Added fats were: diet 1, dogfish liver oil only; diet 2, dogfish liver oil and herring oils mixed 1:1; diet 3, herring oil only.
[c]Digestibility of lipids is determined by comparing the proportion of lipid and cholestane content of dry weight feed and feces.

or wax esters in all three feces samples (Table 3). These could only be confirmed in the TLC/FID quantitative analysis by overloading, otherwise being at or below the lower limit of detectability (Fig. 3). Unhydrolyzed DAGE were also just barely visible on the plate in the lipids from feces of fish fed diets 1 and 2 but could not be definitely determined in the TLC/FID analysis (Table 3). It must be borne in mind that if the two acyl chains are hydrolyzed off the glycerol the mass of the original DAGE is diminished by two-thirds. Despite this having ~5% of the starting material mass of diet 1 as 1-*O*-alkyl diacylglycerol, it could not be resolved with certainty from the expected MAG by TLC/FID. In Figure 3, for example, the TLC/FID peak at or near the origin was nearly the same size, relative to other peaks, in all three sets of fecal lipids. This component peak of the fecal lipids was presumably mostly made up of MAG since DAGE were not included in diet 3. The FID response of TLC/FID is reduced by three of the carbons being already "oxidized," reducing their contribution for combustion in the flame (23).

FFA. The FFA in the dietary fats (Table 3) were so minor a proportion of the fat that they can be ignored when looking at the total picture. They are, however, the dominant lipid class in feces (Fig. 3). Table 3 also shows feces lipids from diet 3 having the lowest proportion of FFA, which in fact show a decrease from diet 1 through diet 2 to diet 3. Conversely there are offsetting increases in other lipid classes except polar lipids which also decrease. This argues for more extensive absorption of both FFA and polar lipids by the salmon receiving a diet supplemented with TAG only. The total lipid recoveries from feces were, respectively, 6.9 ± 0.7, 6.2 ± 0.08, and 6.3 ± 0.04 % (w/w) for diets 1, 2, and 3, somewhat in agreement with this suggestion.

The fatty acid compositions of the fecal FFA are given in Table 5 and may be compared with the total dietary fatty acids and the total fecal fatty acids of Table 2. There are no major differences that can be related to the digestive process and most numbers reflect those of the diets.

Digestion of TAG. It was unexpected to find intact TAG at approximately 10% of fecal lipids (Table 3), and of some interest to try and find compositional details revealing resistance to enzyme attack. Comparison of the dietary TAG with those recovered from the feces (Table 6) shows higher proportions of 20:1 and 22:1 fatty acids in the fecal TAG. These

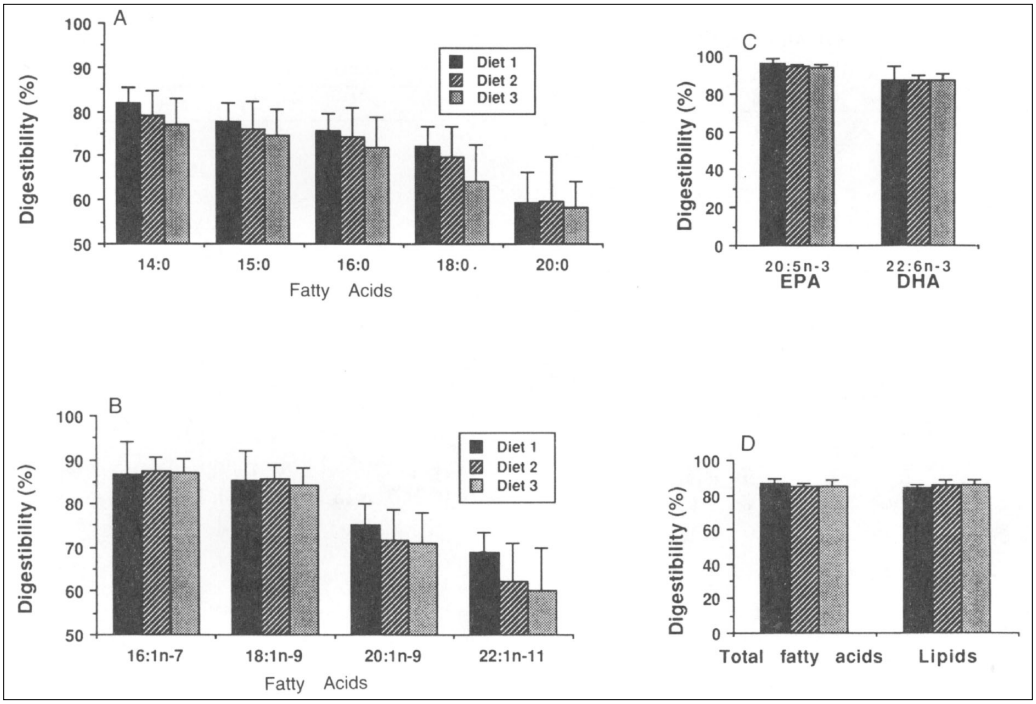

FIG. 1. Digestibility by Atlantic salmon of (A) saturated fatty acids, (B) monounsaturated fatty acids, (C) EPA and DHA, and (D) total fatty acids and total lipids in different diets.

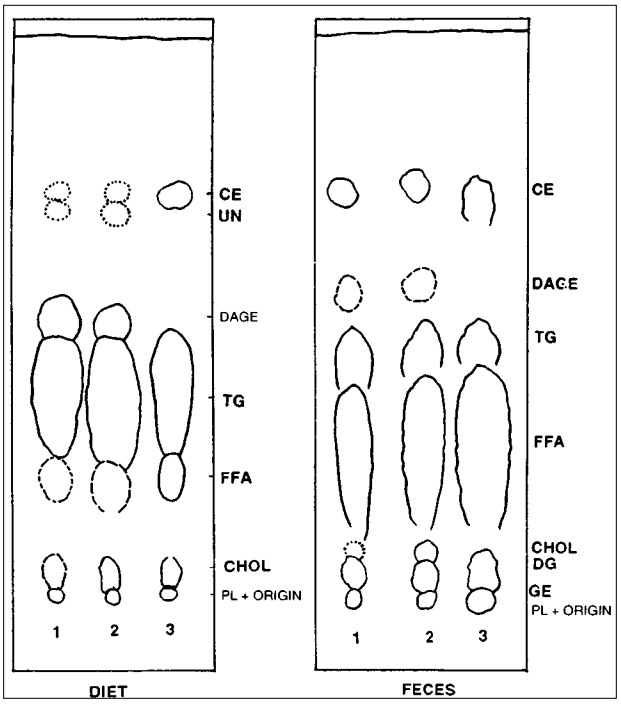

FIG. 2. Thin-layer chromatograms of lipids of diets and feces. TLC plate, soft layer Adsorbosil Plus-1, 20 cm × 20 cm. Solvent, hexane/diethyl ether/acetic acid (85:15:1, by vol). CE, cholesteryl ester; DAGE, diacyl glyceryl ether; TG, triacylglycerols; CHOL, cholesterol; FFA, free fatty acid; GE, glyceryl ether; PL, polar lipids; DG, diacylglycerol; UN, unknown; 1, diet 1; 2, diet 2; 3, diet 3.

TABLE 5
Principal and Biochemically Interesting Free Fatty Acids (w/w%)[a] in Feces Recovered from Atlantic Salmon Fed Different Diets[b]

Fatty acid	Diet 1	Diet 2	Diet 3
14:0	2.31 ± 0.01	4.74 ± 0.09	3.90 ± 0.09
16:0	13.36 ± 0.06	9.56 ± 0.12	8.63 ± 0.26
18:0	2.42 ± 0.01	1.31 ± 0.05	1.02 ± 0.00
Total saturated	19.3	17.0	14.58
16:1n-7	3.75 ± 0.02	3.95 ± 0.09	3.59 ± 0.08
18:1n-9	10.72 ± 0.02	11.56 ± 0.01	5.86 ± 0.11
18:1n-7	2.56 ± 0.02	2.56 ± 0.03	1.35 ± 0.09
20:1n-(9+11)	16.75 ± 0.05	16.53 ± 0.06	18.75 ± 0.07
20:1n-7	1.54 ± 0.02	1.01 ± 0.01	0.51 ± 0.03
22:1n-(11+13)	24.67 ± 0.45	24.88 ± 0.31	37.61 ± 2.98
22:1n-9	4.72 ± 0.11	2.72 ± 0.07	2.07 ± 4.20
Total monounsaturated	68.5	65.9	72.9
18:2n-6	1.05 ± 0.00	1.43 ± 0.01	0.83 ± 0.03
18:3n-3	0.36 ± 0.00	0.59 ± 0.00	0.33 ± 0.02
18:4n-3	0.28 ± 0.01	0.83 ± 0.01	0.46 ± 0.01
20:4n-6	0.21 ± 0.02	0.32 ± 0.00	0.16 ± 0.09
20:5n-3	1.03 ± 0.04	2.38 ± 0.01	1.51 ± 0.03
22:5n-6	0.33 ± 0.01	0.25 ± 0.03	0.26 ± 0.09
22:5n-3	0.74 ± 0.00	0.93 ± 0.03	0.50 ± 0.09
22:6n-3	4.53 ± 0.02	4.63 ± 0.12	2.25 ± 0.14
Total polyunsaturated	12.0	14.8	8.7

[a]Mean and standard deviation of two samples with analyses in duplicate.
[b]Added fats were: diet 1, dogfish liver oil only; diet 2, dogfish liver oil and herring oils mixed 1:1; diet 3, herring oil only.

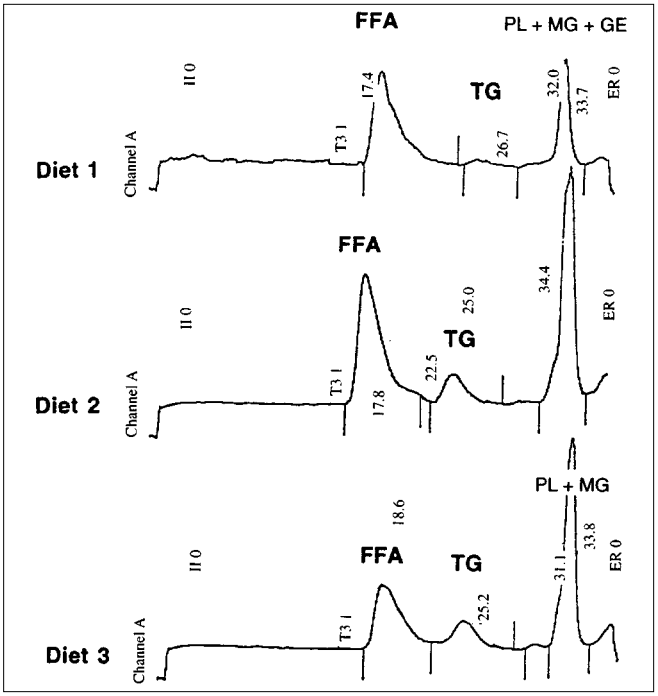

FIG. 3. TLC/FID chromatograms of the lipid classes in feces of Atlantic salmon fed different diets: diet 1 = dogfish liver oil; diet 2 = a mixture of dogfish liver oil and herring oil; diet 3 = herring oil. For operating conditions see text.

fatty acids are probably located in the *sn*-3 position on the glycerol (27), usually considered a focal point for attack by pancreatic lipase (37,38). There are, however, considerable reductions in the longer-chain polyunsaturated acids which formerly were thought to be principally located in the *sn*-2 position. It is now known that in fish oils these fatty acids can also be found in the *sn*-3 (and *sn*-1) positions on a modest proportion of the mixed TAG of fish oils (38). If so, they could have a positive effect on enzyme activity. A particularly interesting point is that 20:5n-3 is reduced much more than 22:6n-3 in the recovered fecal triglycerides. This fits the greater digestibility of the 20:5n-3 (Fig. 1C). Whether this is meaningful in terms of the effect of the position of the ethylenic bond nearest the carboxyl group (39) has led to much speculation (40), but the unequal distribution on glycerol may be a more likely reason. Our work with the DAGE may cast some light on this and other problems. For example, one hypothesis could be that the addition of the 1-*O*-alkyl DAG to diets 1 and 2 could lead to more persistent micelles in the digestive system, carrying more TAG along to the feces. Table 3 shows exactly the opposite effect, with MAG, DAG, and TAG all being highest in feces from diet 3 which was free of the DAG ethers. The higher proportion of long-chain monoenes may be a better explanation in this particular case.

Fatty acid composition of dietary 1-O-*alkyl DAGE*. The dogfish liver oil DAGE in diets 1 and 2 were recovered and examined for both acyl groups and the 1-*O*-alkylglycerol ester. Since the same oil was simply diluted, this fatty acid analysis provided a check on the methods of analysis and with the exception of 14:0, subject in most lipid procedures to losses from solubility and volatility (41) the results were reasonably coherent. A surprising feature (Table 7) was the high proportion (≤50%) of 22:5n-3 compared to 22:6n-3. This relationship is usually a property of oils from marine mammals (37,42). The TAG from the dogfish liver oil and the Atlantic herring oil showed the more usual ratio of about 1 part of 22:5n-3 to 5 parts of 22:6n-3 (Table 6). The 20:5n-3 of the fatty acids of DAGE seemingly was reduced relative to the amount in the TAG, with the total for these two pentaenoic fatty acids being approximately equal to the 22:6n-3 in DAGE both before and after passage through the fish. The conversion of 20:5n-3 to 22:6n-3 is now thought to go through 22:5n-3, 24:5n-3, and 24:6n-3 (43). Since the GE are biosynthesized in the dogfish liver, there seems to be a degree of specificity for the DAGE of a failed attempt to increase 22:6n-3. The corresponding TAG acyl groups are closer to the norm for fish oils (44,45) and presumably reflect dietary fatty acid intake.

Digestion of DAGE. Unexpectedly the 16:1n-7 was increased two or threefold in the fatty acids of the DAGE recovered from the feces (Table 8), compared to the diet figures (Table 7), possibly also leading to a slight enrichment of 18:1n-7 relative to 18:1n-9. Unlike fecal TAG, both 20:1 and 22:1 were slightly lower in the fecal DAGE than in the diet DAGE. The figures for the polyunsaturated acids did not agree very well for the fecal DAGE from the two diets (Table 8). However the 22:5n-3, relative to 22:6n-3, was rather high for fish oils in both DAGE. Both 20:4n-6 and 20:5n-3 may have resisted digestive hydrolysis (*cf.* Table 7). By including 18:4n-3 in these comparisons, it seems likely that in DAGE polyunsaturated fatty acids with ethylenic bonds in the Δ4 (22:6n-3), (22:5n-6), Δ5 (20:5n-3), (20:4n-6), and Δ6 (18:4n-3) positions resist hydrolysis, while that with the bond in the Δ7 position (22:5n-3) is more readily hydrolyzed by Atlantic salmon digestive enzymes. In the TAG (Table 6), the 22:5n-6 (Δ7) seemed to resist hydrolysis compared to 18:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3. This view is within the polyunsaturated groups which otherwise were well absorbed (Table 4).

1-O-*Alkylglycerol ethers*. Tables 7 and 8 also provide a comparison of the glyceryl ether skeletons of the DAGE. The original composition is typical of marine GE (10,45). Compared to the triglycerides, FFA, and diacyl fatty acids of the DAGE, the 16:1n-7 in the alkyl chains seem to be very high (Tables 7 and 8), but does not affect the 18:1n-9/18:1n-7 ratio. An unusual feature not previously reported for marine GE is the isomer compositions of the 20:1 chains. The 20:1n-7 isomer exceeds the 20:1n-9 isomer. As expected, the 20:1n-9 was higher in each case than the adjacent 20:1n-11 shoulder (Table 7, not shown separately) but actual numbers for n-11, n-9, and n-7 were 0.50, 2.31, 5.20 for diet 1; 0.35, 3.35, 6.55 for diet 2. The proportions of all components, including these isomers (not shown in detail) were very similar in the alkyl chains of the DAGE recovered from the feces. Other than the

TABLE 6
Principal and Biochemically Interesting Fatty Acids (w/w%)[a] in Triacylglycerols of Diets[b] Fed to Atlantic Salmon and of Those in Feces Recovered from Atlantic Salmon

	Diet fed			Feces		
Fatty acid	Diet 1	Diet 2	Diet 3	Diet 1	Diet 2	Diet 3
14:0	3.55 ± 0.22	6.61 ± 1.91	7.03 ± 1.14	2.71 ± 0.02	4.92 ± 0.65	7.20 ± 0.11
16:0	12.60 ± 4.45	12.37 ± 1.10	9.86 ± 0.95	10.14 ± 0.4	49.27 ± 1.34	10.13 ± 0.11
18:0	3.42 ± 0.36	1.68 ± 0.17	0.78 ± 0.10	1.87 ± 0.33	1.21 ± 0.15	0.87 ± 0.03
Total saturated	21.6	22.2	19.4	16.7	17.1	20.5
16:1n-7	5.69 ± 1.25	6.58 ± 0.45	5.87 ± 0.35	4.27 ± 0.22	5.72 ± 0.80	5.21 ± 0.07
18:1n-9	11.32 ± 3.50	9.24 ± 1.85	6.38 ± 1.55	10.80 ± 0.19	9.86 ± 0.70	5.14 ± 0.04
18:1n-7	3.30 ± 0.70	2.24 ± 0.45	1.65 ± 0.55	2.70 ± 0.19	2.47 ± 0.15	1.54 ± 0.02
20:1n-(9+11)	15.72 ± 0.87	16.10 ± 1.12	17.69 ± 0.61	17.95 ± 0.04	20.58 ± 0.35	19.05 ± 0.07
20:1n-7	1.03 ± 0.10	0.77 ± 0.02	0.50 ± 0.04	1.47 ± 0.05	1.00 ± 0.01	0.51 ± 0.01
22:1n-(11+13)	18.35 ± 2.55	20.60 ± 1.55	28.12 ± 9.05	27.10 ± 0.66	28.08 ± 0.40	32.46 ± 0.18
22:1n-9	2.35 ± 0.14	1.85 ± 0.13	1.35 ± 0.50	4.39 ± 0.05	2.42 ± 0.65	1.58 ± 0.47
Total monounsaturated	60.0	59.4	63.9	73.2	73.6	68.3
18:2n-6	1.08 ± 0.10	1.22 ± 0.15	1.01 ± 0.02	1.49 ± 0.02	1.02 ± 0.00	0.74 ± 0.02
18:3n-3	0.60 ± 0.03	0.76 ± 0.01	0.62 ± 0.02	0.31 ± 0.05	0.46 ± 0.08	0.43 ± 0.01
18:4n-3	0.91 ± 0.02	1.70 ± 0.03	1.55 ± 0.4	50.24 ± 0.01	0.45 ± 0.01	0.75 ± 0.01
20:4n-6	0.24 ± 0.01	0.19 ± 0.01	0.20 ± 10.02	0.15 ± 0.03	0.15 ± 0.03	0.14 ± 0.02
20:5n-3	3.65 ± 0.15	4.57 ± 0.65	4.38 ± 0.26	0.67 ± 0.00	0.94 ± 0.09	2.21 ± 0.02
22:5n-6	0.21 ± 0.03	0.16 ± 0.02	0.47 ± 0.05	0.42 ± 0.07	0.19 ± 0.02	0.32 ± 0.01
22:5n-3	1.20 ± 0.02	0.80 ± 0 05	0.73 ± 0.05	0.50 ± 0.10	0.39 ± 0.01	0.54 ± 0.01
22:6n-3	5.60 ± 0.95	4.40 ± 0.10	3.26 ± 0.11	2.38 ± 0.33	2.26 ± 0.10	2.67 ± 0.04
Total polyunsaturated	18.5	18.3	16.9	9.8	9.3	11.0

[a]Mean and standard deviation of two analyses.
[b]Added fats were: diet 1, dogfish liver oil only; diet 2, dogfish liver oil and herring oils mixed 1:1; diet 3, herring oil only.

presumed origin in 16:1n-7, no explanation can be offered for this proportion of 20:1n-7 in the GE alkyl chains. Unfortunately Lie and Lambertsen (12) do not include these details. The 22:1 alkyl chains were very minor components and isomer ratios not very reliable. The C_{18} polyunsaturated alkyl chains of the GE in the diets (Table 7) did not appear in the DAGE of the feces, but were observed in the GE of the feces (Table 8). This difference is likely an artifact of manipulative oxidation of the DAGE and is not considered metabolically important. Digestion did not obviously affect the GE alkyl chain composition.

Serum and muscle lipids. Major lipid class results for diet, feces, chylomicron, and muscle are compared in Figure 4. The analysis of the recovered chylomicrons (Tables 3 and 9, Fig. 4) showed that TAG and DAGE were absorbed and circulated in chylomicrons roughly in accord with proportions in the diet. This did not influence the TAG, sterols, or sterol esters, but may have had some slight effect on the serum polar lipids. Given the large standard deviation of data for this lipid class (Table 3), such an effect may be an artifact of the method.

The fatty acids of the chylomicron lipids were almost identical for all three diets, so only those for the fish on diet 1 are presented (Table 9). The total lipid fatty acids would be influenced by the fatty acids of the stable polar lipids, presumably high in 22:6n-3. The TAG did not show a composition very close to the dietary total fatty acids of Table 2, presumably having been selectively hydrolyzed from original TAG positions and reassembled with the inclusion of fatty acids already circulating in the fish. Probably different lipids were hydrolyzed and absorbed at different points in the intestine, and some fatty acids (saturated, monoethylenic) could be biosynthesized by the salmon. The fatty acids of the chylomicron DAGE showed a strong emphasis on saturated fatty acids, mostly at the expense of the monoethylenic fatty acids, and were also depleted in polyunsaturated fatty acids compared

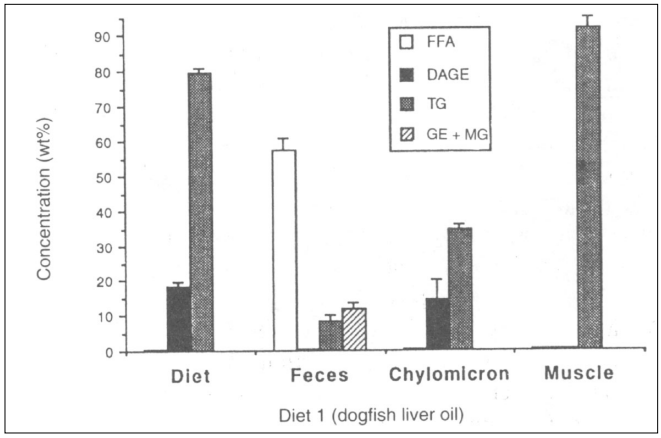

FIG. 4. Lipid class composition of diet, feces, chylomicrons, and muscle of fish fed diet 1 (dogfish liver oil).

TABLE 7
Principal and Biochemically Interesting Components (w/w%) of 1-*O*-Alkyl Diacylglycerols in Diets 1 and 2 Fed to Atlantic Salmon

	Diet 1		Diet 2	
Fatty acid	Fatty acids	1-*O*-alkyl ether	Fatty acids	1-*O*-alkyl ether
14:0	1.19 ± 0.16	5.24 ± 0.85	2.30 ± 0.35	5.09 ± 0.20
16:0	13.14 ± 1.15	12.37 ± 0.25	15.46 ± 0.60	12.93 ± 0.60
18:0	1.43 ± 0.05	5.53 ± 0.25	1.43 ± 0.20	6.31 ± 0.92
Total saturated	17.1	28.0	21.2	29.5
16:1n-7	2.08 ± 0.20	11.25 ± 0.65	2.79 ± 0.01	10.52 ± 0.30
18:1n-9	8.82 ± 0.70	39.19 ± 1.17	8.79 ± 0.01	34.68 ± 7.35
18:1n-7	1.62 ± 0.10	4.92 ± 0.11	1.38 ± 0.45	4.57 ± 0.95
20:1n-(9+11)	18.02 ± 3.65	2.81 ± 0.26	15.79 ± 2.55	3.70 ± 0.18
20:1n-7	1.12 ± 0.45	5.20 ± 0.60	1.15 ± 0.50	6.55 ± 0.10
22:1n-(11+13)	26.74 ± 6.60	0.75 ± 0.03[c]	24.13 ± 4.01	1.01 ± 0.08[c]
22:1n-9	5.26 ± 0.15	n.d.	5.34 ± 0.34	n.d.
Total monounsaturated	68.4	69.5	63.4	65.8
18:2n-6	0.60 ± 0.45	1.94 ± 0.25	0.67 ± 0.01	1.91 ± 0.04
18:3n-3	0.39 ± 0.03	0.32 ± 0.01	0.39 ± 0.01	0.32 ± 0.01
18:4n-3	0.18 ± 0.05	n.d.	0.35 ± 0.02	n.d.
20:4n-6	0.10 ± 0.14	n.d.	0.14 ± 0.02	n.d.
20:5n-3	1.11 ± 0.10	n.d.	1.64 ± 0.50	n.d.
22:5n-6	0.41 ± 0.11	n.d.	0.65 ± 0.18	n.d.
22:5n-3	3.14 ± 0.19	n.d.	2.48 ± 0.22	n.d.
22:6n-3	4.48 ± 0.18	n.d.	4.95 ± 0.15	n.d.
Total polyunsaturated	14.99	2.67	15.66	2.40

[a]Mean and standard deviation of two samples with analyses in duplicate; n.d., not detected. [b]Added fats were: diet 1, dogfish liver oil only; diet 2, dogfish liver oil and herring oils mixed 1:1; diet 3, herring oil only. [c]Total 22:1.

TABLE 8
Principal and Biochemically Interesting Components (w/w%)[a] of 1-*O*-Alkyl Diacylglycerols Recovered from Feces of Atlantic Salmon Fed Diets 1 and 2

	Diet 1			Diet 2		
	Total DAGE		Free	Total DAGE		Free
Fatty acid	Fatty acids	1-*O*-alkyl ether	GE	Fatty acids	1-*O*-alkyl ether	GE
14:0	4.99 ± 0.80	5.22 ± 0.02	4.83	5.19 ± 0.87	6.24 ± 0.87	4.75
16:0	13.73 ± 0.27	8.69 ± 1.25	8.78	14.49 ± 3.84	10.29 ± 0.93	9.64
18:0	1.89 ± 0.30	5.03 ± 0.56	4.20	0.94 ± 0.10	3.61 ± 0.08	5.12
Total saturated	23.5	24.7	25.1	25.0	26.6	25.9
16:1n-7	5.11 ± 0.50	11.21 ± 1.05	10.00	8.51 ± 0.26	7.59 ± 0.30	9.31
18:1n-9	9.38 ± 0.29	39.65 ± 5.20	37.58	10.74 ± 0.65	40.23 ± 0.62	35.77
18:1n-7	2.62 ± 0.60	5.13 ± 0.53	5.28	2.32 ± 0.30	4.78 ± 0.14	7.65
20:1n-(9+11)	14.28 ± 2.95	3.42 ± 0.08	3.41	14.49 ± 0.23	2.26 ± 0.14	3.02
20:1n-7	1.37 ± 0.20	6.51 ± 0.75	9.45	0.70 ± 0.50	6.08 ± 0.31	7.63
22:1n-(11+13)	20.97 ± 0.54	1.14 ± 0.02[c]	1.33[c]	19.82 ± 0.60	1.79 ± 1.44[c]	1.04[c]
22:1n-9	4.43 ± 0.40	—	—	1.67 ± 0.10	—	n.d.
Total monounsaturated	61.5	72.6	72.5	61.6	69.2	70.1
18:2n-6	1.01 ± 0.02	n.d.	0.49	1.44 ± 0.02	n.d.	0.31
18:3n-3	0.51 ± 0.01	n.d.	0.31	0.68 ± 0.01	n.d.	0.39
18:4n-3	0.62 ± 0.05	n.d.	n.d.	1.40 ± 0.02	n.d.	0.32
20:4n-6	0.21 ± 0.02	n.d.	n.d.	0.22 ± 0.02	n.d.	n.d.
20:5n-3	2.03 ± 0.07	n.d.	n.d.	1.98 ± 0.05	n.d.	n.d.
22:5n-6	0.32 ± 0.04	n.d.	n.d.	0.19 ± 0.08	n.d.	n.d.
22:5n-3	1.02 ± 0.04	n.d.	n.d.	0.88 ± 0.21	n.d.	n.d.
22:6n-3	3.56 ± 0.25	n.d.	n.d.	1.70 ± 0.08	n.d.	n.d.
Total polyunsaturated	15.0	2.6	2.6	13.3	4.4	2.4

[a]Mean and standard deviation of two sample analyses analysed in duplicate except for free GE; n.d., not detected. [b]Added fats were: diet 1, dogfish liver oil only; diet 2, dogfish liver oil and herring oils mixed 1:1; diet 3, herring oil only. [c]Total 22:1.

TABLE 9
Principal Fatty Acid Chains (w/w%) of Some Chylomicron Lipids from Fish on Diet 1, and the Alkyl Chains of 1-*O*-Alkyl DAGE

	Fatty acids			
Chain structure	Total lipid	TAG	DAGE	1-*O*-alkyl ether
14:0	3.00	3.07	11.36	3.51
16:0	19.07	9.75	25.34	15.74
18:0	3.04	3.24	3.78	3.21
Total saturated	26.7	19.7	46.4	28.8
16:1n-7	3.67	5.11	5.46	7.26
18:1n-9	17.81	23.24	11.46	36.45
18:1n-7	2.32	3.33	2.36	4.03
20:1n-(9+11)	4.92	9.42	5.98	2.57
20:1n-7	0.38	0.82	0.58	8.79
22:1n-(11+13)	3.46	6.88	5.53	8.30[a]
22:1n-9	0.64	1.99	1.08	—
Total monounsaturated	35.7	54.5	37.5	67.8
18:2n-6	2.52	2.47	1.81	1.01
18:3n-3	0.32	0.55	0.65	1.48
18:4n-3	0.40	0.78	1.23	n.d.
20:4n-6	0.83	0.30	0.20	n.d.
20:5n-3	6.46	4.59	1.76	n.d.
22:5n-6	0.22	0.44	0.10	n.d.
22:5n-3	2.21	3.24	0.62	n.d.
22:6n-3	20.30	6.79	2.19	n.d.
Total polyunsaturated	37.7	25.7	16.1	2.68

[a]Includes 22:1n-9; TAG, triacylglycerols. See Table 3 for other abbreviation.

to those of the TAG. These details suggest selective hydrolysis of the original structure, but it is not known if this is an effect in the intestine or in the intestinal wall, where absorption of residual 1-*O*-alkylglycerol might be expected or on reesterification as proposed by Lie and Lambertsen (12) for cod. A noteworthy feature of the 1-*O*-alkyl GE of the chylomicron DAGE is the inclusion of a peak in the position for 20:1n-7. This component was also apparent in the Tables 7 and 8 results for the original and fecal DAGE and GE. Probably the 18:1n-7 alkyl chain is also increased in the fecal GE relative to 18:1n-9, lending support to the proposed structure 20:1n-7. Compared to dietary GE (Table 7), 22:1 GE was unexpectedly high in the chylomicrons. In cod chylomicrons, the GE 20:1 was elevated relative to diet, but was reported as 20:1n-11 while 22:1 was not listed (12).

The muscle lipids were dominated by TAG to the extent that direct measurement of DAGE by TLC/FID was not practical. Accordingly the total muscle lipid was saponified and the unsaponifiable were recovered. The extremely low uptake of DAGE by muscle is reassuring. Many highly regarded fish and shellfish food products contain low levels of either 1-*O*-alkyl- or 1-*O*-alkenyldiacylglycerols (e.g., 11), and it is evident that the traces in salmon muscle would not be objectionable.

Conclusions. Overall, we anticipated that the liver oil of Atlantic dogfish would be similar to that of the Pacific dogfish, widely regarded as the same species (46). That oil also contained DAGE and included 10–15% longer-chain n-3 fatty acids that could be liberated by saponification of both TAG and DAGE (36). Atlantic dogfish liver oil is a very underutilized by-product of one of the few remaining fish stocks in the Atlantic coast of Canada and the United States. It has 1-*O*-dialkylglycerol ethers with industrial potential (47). However the TAG fatty acid composition is virtually the same as that of herring oil, providing sufficient longer chain ω-3 fatty acids for the growth and health of farmed Atlantic salmon. The fatty acids of the DAGE are similar and are liberated by digestive enzymes in this species and add to the supply. The residual 1-*O*-alkylglycerol ethers are adsorbed and circulated to a limited extent but are not deposited to any extent in the edible muscle of the salmon. Thus, dogfish liver oil should be entirely satisfactory as a lipid source for incorporation in Atlantic salmon feeds.

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Influence of Diet on the Kinetic Behavior of Hepatic Carnitine Palmitoyltransferase I Toward Different Acyl CoA Esters

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ABSTRACT: The influence of diet on the kinetics of the overt form of rat liver mitochondrial carnitine palmitoyltransferase (CPT I; EC 2.3.1.21) was studied using rats fed either a low-fat diet (3% w/w fat), or diets which were supplemented with either olive oil (OO), safflower oil (SO) or menhaden (fish) oil (MO) to 20% w/w of fat (high fat diets). When animals were fed each of these four diets for 10 days, the order of the apparent maximal activity (v_{max}) of CPT I toward various individual fatty acyl CoA, when measured under a fixed molar ratio of acyl CoA/albumin, was 16:1n-7>18:1n-9 >18:2n-6>16:0>22:6n-3, and was thus not affected by the fat composition of the diet. However, in all but one case, the SO and MO diets elicited a higher v_{max} for each substrate than either the LF diet or the high fat OO diet. The apparent $K_{0.5}$ for the different acyl CoA esters was generally lowest in LF-fed animals, and highest in those fed the high-fat SO diet. Moreover, when compared with the situation of animals fed high-fat diets, the $K_{0.5}$ values of CPT I in LF-fed animals for palmitoyl CoA and oleoyl CoA were low. This possession by CPT I of a high "affinity" toward these nonessential fatty acyl CoAs, but a lower "affinity" toward linoleoyl CoA, the ester of an essential fatty acid, may enable this latter fatty acid to be spared from oxidation when its concentration in the diet is low. The data also emphasize that palmitoleoyl CoA, if available in the diet, is likely to be utilized by CPT I at a high rate.
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The overt form of carnitine palmitoyltransferase (CPT I; EC 2.3.1.21) catalyzes the reaction that commits to oxidation, within the mitochondria, the long-chain acyl CoA esters that are formed in the cytosol (1), and thus occupies a key regulatory step in the metabolism of long-chain fatty acids. CPT I has been shown to display different kinetic properties toward different acyl CoA, thereby determining the extent to which these compounds serve as substrates for β-oxidation (2,3). Since the maximal activity (v_{max}) and the substrate concentration at which one-half maximal activity is attained ($K_{0.5}$) differ for individual acyl CoA, the ultimate flux through CPT I to β-oxidation *in vivo* is determined by the relationship between the relative maximal activity and affinity of this enzyme for each potential substrate.

Although there have only been a few studies of the substrate selectivity of CPT I, it is evident that this selectivity can differ markedly between species. For example, whereas the relative activity of rat liver CPT I is 18:3n-3>18:2n-6> 20:5n-3≈16:0>20:4n-6>18:1n-9>22:6n-3>18:0 at 10 μM (2), that of the Antarctic fish *Notothenia* sp. is 16:1n-7> 20:5n-3>18:2n-6≈18:1n-9>22:6n-3>20:4n-6>18:3n-3 (3). Moreover, the relative activity of this enzyme toward C_{22} polyunsaturated acyl CoA of the n-3 series is low in the rat (2), but relatively high in *Notothenia* sp. (3). Likewise, in comparison with the substrate preference of CPT I in rat liver, the activity of this enzyme in *Notothenia* sp. is also relatively high toward other marine-type fatty acids such as 16:1n-7 and 20:5n-3, but relatively low toward 18:3n-3.

Evidence that CPT I displays a marked difference in its kinetic behavior toward various acyl CoA and that the pattern of substrate preference differs between species whose diets are qualitatively different suggests that the kinetic characteristics of this enzyme may be determined by the fatty acid composition of the diet. This could be achieved either through the expression of enzyme more favorably disposed to particular fatty acyl CoA, or by changes to the biophysical properties of the outer mitochondrial membrane, in which certain domains of the CPT I protein reside (1).

Previous work has shown that the v_{max} of CPT I toward palmitoyl CoA and the sensitivity of this enzyme to inhibition by malonyl CoA are influenced profoundly by the fatty acid composition of the diet (4). It also demonstrated that, of a range of different high-fat diets, the one which was supplemented with safflower oil to 20% w/w of fat encouraged highest CPT I activity toward palmitoyl CoA. Since diet directly influences the composition of the cytosolic acyl CoA pool available to CPT I in the liver, it might be advantageous if the kinetics of this enzyme were able to reflect a preference for the predominant types of fatty acid in the diet, thus regulating the types of fatty acids that are oxidized when the relative

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Abbreviations: BSA, bovine serum albumin; CPT I, overt form of mitochondrial carnitine palmitoyltransferase, $K_{0.5}$, total substrate concentration at which one-half maximal velocity (v_{max}) is attained; LF, low fat; MO, menhaden oil; OO, olive oil; SO, safflower oil.

proportions and/or tissue content of the fatty acids change. The influence of certain fat diets on the level of CPT I activity in the liver, which determines the partitioning of acyl CoA between oxidation and esterification, has been examined recently by Moir *et al.* (5). By employing the elegant procedure of labeling the acyl moiety of cholesteryl esters incorporated into apoC-depleted lipoproteins (6), Moir *et al.* (5) encouraged a labeled acyl group ([1-^{14}C]-oleate) to be taken up selectively by the liver. Their results showed that the partitioning of the acyl group was diet-dependent. However, the extent to which these differences are determined by the specific kinetic properties of CPT I at the "branchpoint" between oxidation and esterification is yet to be established.

Thus, the present study has examined the influence of diet on the kinetic behavior of CPT I using acyl CoA esters of a range of fatty acids which differed in chain length and/or the extent of unsaturation. The acyl CoA were synthesised enzymatically by employing the acyl CoA synthetase method of Crockett and Sidell (3), and used subsequently as substrates for estimating CPT I activity by the standard radiochemical assay (7). However, a constant 6.1:1 molar ratio of acyl CoA/albumin was adopted to enable the apparent ν_{max} and $K_{0.5}$ values to be determined more accurately (see below for rationale). Comparisons between the maximal activities and $K_{0.5}$ values of CPT I toward different acyl CoA substrates, in animals maintained on different diets, indicate that the kinetic behavior of this key regulatory enzyme is influenced by the fatty acid composition of the diet.

TABLE 1
The Fatty Acid Composition of the Experimental Diets[a]

	Composition of the diets (mol%)			
Fatty acids	LF	OO	SO	MO
12:0	2.2	—	2.2	—
14:0	2.4	—	0.4	10.9
16:0	31.6	11.0	11.8	24.7
16:1n-7	—	—	—	16.9
18:0	7.4	3.9	4.4	4.4
18:1n-9	21.1	69.9	20.8	13.8
18:2n-6	31.5	15.2	60.4	5.5
18:3n-3	—	—	—	2.3
18:3n-6	—	—	—	—
20:5n-3	—	—	—	11.7
22:6n-3	—	—	—	5.2
Others	3.8	—	—	4.6

[a]Lipid from a sample of each diet was extracted using chloroform/methanol (2:1, vol/vol). Fatty acids were prepared by saponification in methanolic KOH and extracted into ethyl acetate. Fatty acids were then methylated using diazomethane, and the fatty acid methyl esters were analyzed by gas chromatography as described previously (4). Data are the mean of two determinations. (—) indicates not detected. Abbreviations: LF, low-fat control diet; OO, olive oil diet; SO, safflower oil diet; and MO, menhaden oil diet. Where a fatty acid was detected at less than 0.2 mol%, it was included in the minor category "Others."

MATERIALS AND METHODS

Animals and diets. Male weanling Wistar rats (40–50 g) were purchased from the Animal Resource Centre (Perth, Western Australia) and housed under temperature-controlled conditions. They were exposed to a 12-h light–dark cycle and provided for 10 d with one of four experimental diets.

The low-fat (LF) semisynthetic diet contained 3% w/w basic fat (1% beef tallow + 1% safflower oil + 1% corn oil), 52% w/w carbohydrate (20.8% corn starch + 31.2% sucrose), 16% w/w protein (sodium caseinate), and 29% w/w nondigestible material (cellulose). The three high-fat diets were supplemented to a total of 20% w/w fat, using either olive oil (OO), safflower oil (SO), or menhaden oil (MO). The high-fat diets contained the same amounts of carbohydrate, basic fat and protein, but the amount of nondigestible material was lowered proportionately to accommodate the increased fat content. The fatty acid composition of LF, OO, SO, and MO diets are given in Table 1. All diets contained 1.2 g · kg^{-1} vitamin E as an antioxidant, and adequate amounts of vitamins and minerals. The weight gain and food intake of all animals were monitored throughout the experimental period and did not differ significantly between animals on each of the four diets. Animals were killed by CO_2 asphyxiation.

Chemicals. The L-[methyl ^{3}H]-carnitine hydrochloride was supplied by the Radiochemical Centre (Amersham, Buckinghamshire, United Kingdom). Oleic acid (sodium salt), linoleic acid (sodium salt), palmitoleic acid (free acid), docosahexaenoic acid (free acid), palmitoyl CoA, and all other biochemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). The sucrose and butanol were supplied by BDH Chemicals (Poole, United Kingdom), and the acyl CoA synthetase (*Pseudomonas* spp.) by Boehringer (Mannheim, Germany).

*Synthesis of fatty acid salts***.** Since fatty acid salts were required for the enzymatic synthesis of acyl CoA esters (3), the fatty acids that were unavailable commercially as salts (palmitoleic acid and docosahexaenoic acid) were converted into their corresponding potassium salts by reacting them with a 30% molar excess of potassium (as 0.1M KOH) at 60°C under a gentle stream of nitrogen. For example, in the case of palmitoleic acid, the fatty acid was dissolved in ethanol (10 mg/mL) with gentle mixing, and a 0.016 mL aliquot (0.16 mg) removed and added to 0.5 mL ethanol plus 0.020 mL 0.1 M ethanolic KOH. The solvents were evaporated under a gentle stream of nitrogen, and the dry salt of the fatty acid was stored in a sealed vial under nitrogen at −80°C until use (within 24 h).

Synthesis of acyl CoA esters. Acyl CoA esters were synthesized by a modification of the method of Crockett and Sidell (3). The reaction mixtures for the syntheses contained 0.62 mM sodium or potassium salt of a fatty acid, 0.7 mM CoASH, 5 mM ATP, 20 mM $MgCl_2$, 0.5 units acyl CoA synthetase/mL (1 unit = 1 µmol of product formed/min) in 150 mM Tris-HCl (pH 7.8 at 25°C). (Subsequently it was found that when fatty acids were converted to their potassium salt immediately prior to esterification they were more readily esterified to their corresponding acyl CoA.) Butylated hydroxytoluene (BHT) was included in the synthesis at 0.005% wt/vol to prevent oxidation of the unsaturated acyl chains. The syn-

theses were conducted at 25°C, with occasional, gentle agitation to maintain complete solubilization of the fatty acids.

The extent to which each fatty acid salt was esterified was monitored periodically by following the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) by residual CoASH at 412 nm in samples of the reaction mixture as described by Bar-Tana *et al.* (8). A 1.5 mM solution of 5,5′-dithiobis-(2-nitrobenzoic acid) was prepared in 150 mM Tris-HCl (pH 7.8 at 25°C). The results of these biosyntheses are given in Figure 1. Once fully converted, each fatty acyl CoA was diluted to 500 µM with a stock bovine serum albumin (BSA) solution (prepared in deionized H_2O) to achieve an acyl CoA/BSA molar ratio of 6.1:1 and then frozen at –80°C until use.

The preparation of mitochondria. Animals, which had been on one of the four experimental diets for 10 d, were killed at 0800 hours at the end of the dark phase. Liver mitochondria were prepared as previously described (4) using a method which substantially reduces contamination by other organelle sources of CPT activity. Protein was measured by the method of Gornall *et al.* (9) but with a 30-min preincubation of all mitochondrial aliquots with 2.0 M NaOH to ensure that the protein was completely solubilized prior to assay.

CPT I assay. The activity of CPT I was measured in the direction of radiolabeled acylcarnitine formation using a procedure similar to that of Grantham and Zammit (7). This method is frequently chosen because of the ease with which the co-substrate (labeled carnitine) can be separated from the reaction product (labeled acylcarnitine), and because the activity of palmitoyl CoA hydrolase does not interfere with this system as much as it does when labeled palmitoyl CoA is used as a tracer (10). Albumin is a necessary ingredient in the assay because it stabilizes the outer mitochondrial membrane by reducing the detergent properties of acyl CoA, thus minimizing any contribution of CPT II to the assay (11). However, in the presence of fixed amounts of albumin in the assay medium, CPT I can demonstrate an activity vs. substrate concentration plot that is sigmoidal because albumin competes with the enzyme for binding to free acyl CoA substrate. The apparent sigmoidicity seen by some workers (10,12) has thus been attributed to substrate binding by albumin (10). At low acyl CoA concentrations, binding sites on albumin may compete very effectively for acyl CoA, resulting in an underestimation of the actual CPT I activity for the total concentration of acyl CoA in the assay medium. At higher concentrations, the binding sites on albumin become saturated, thus "relinquishing" more substrate to CPT I and thereby enabling relatively higher activities to be detected (13). Thus, as acyl CoA concentration increases under conditions of a fixed amount of albumin, increasing CPT I activity is due to increasing ratios of free to bound acyl CoA. It has therefore been concluded that this increasing molar ratio of acyl CoA/albumin, rather than the total concentration of acyl CoA present, is responsible for the sigmoidal relationship (10).

In order to "correct" for this artifactual sigmoidicity, Pauly and McMillin (10) measured CPT I against increasing concentrations of palmitoyl CoA, but at a number of different fixed ratios of palmitoyl CoA/albumin. Since Scatchard analysis has shown that albumin possesses five or six high-affinity binding sites for palmitoyl CoA, a 6.1:1 molar ratio of palmitoyl CoA/albumin was chosen by Pauly and McMillin (10) in order to saturate almost completely the binding capacity of albumin and yield an activity-vs.-substrate concentration plot for CPT I that is hyperbolic. Under these conditions, a Lineweaver-Burk plot is linear, from which a more accurate ν_{max} and $K_{0.5}$ can be determined. However, it must be recognized that, although fixing the molar ratio of acyl CoA to albumin ensures consistent competition between albumin and the enzyme for free acyl CoA, the precise free concentration remains unknown. It is also not known whether the affinity of albumin varies for the different acyl CoA. Consequently, the terminology "apparent ν_{max}" for the maximal activity, and "apparent $K_{0.5}$" for the total substrate concentration at which half-maximal enzyme activity is attained, has been adopted in agreement with other workers (2,10,14).

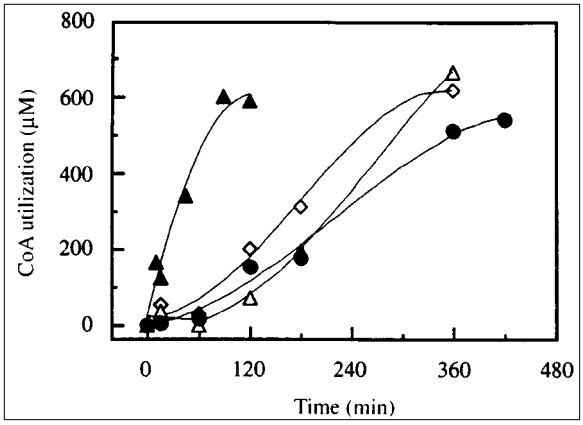

FIG. 1. The utilization of CoA against time by acyl CoA synthetase. Acyl CoA esters were synthesised *in vitro* using purified acyl CoA synthetase from *Pseudomonas* sp. The fatty acid concentration available for esterification to CoA was 0.62 mM in each case. Data represent a single synthesis of each acyl CoA ester by 0.5 Units of enzyme. Acyl CoA esters of the following fatty acids were synthesized: palmitoleic acid (16:1n-7, ▲), oleic acid (18:1n-9, ●), linoleic acid (18:2n-6, △), and docosahexaenoic acid (22:6n-3, ◇). See the Materials and Methods section for a full explanation of the assay system.

RESULTS

Substrate saturation curves for CPT I. Substrate saturation curves for the hepatic CPT I activity, in animals fed either a LF, OO, SO or MO diet and when assayed in the presence of a 6.1:1 molar ratio of acyl CoA/albumin, are presented in Figure 2. CPT I exhibits hyperbolic kinetic behavior over the range of concentrations chosen for each acyl CoA.

The mean ν_{max} for palmitoyl CoA (16:0) decreased in the following order of diets MO>SO>LF>OO (Table 2). The apparent $K_{0.5}$ for palmitoyl CoA of the CPT I of each group of animals decreased in the order SO>MO>OO>LF (Table 3).

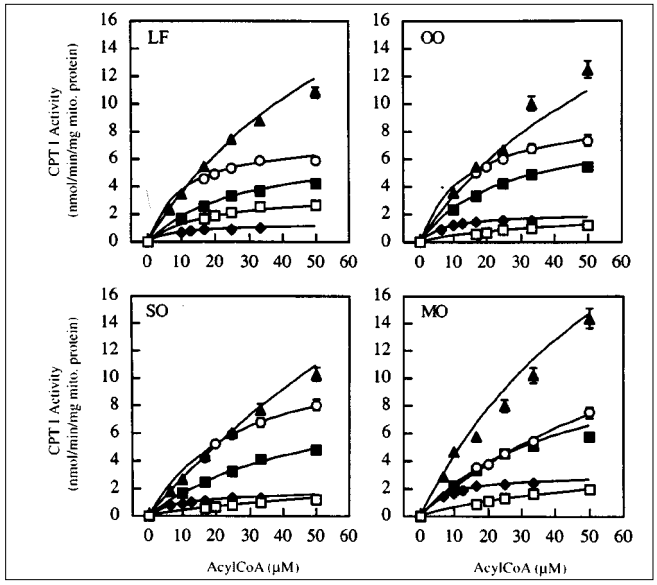

FIG. 2. Liver mitochondrial CPT I activity as a function of acyl CoA concentration. Rats were fed for 10 d on either an LF or one of three high-fat diets, and liver mitochondrial CPT I activity was measured. Michaelis-Menten curves were constructed from the activity of CPT I toward different acyl CoA substrates at a range of concentrations, and at constant ratio (6.1:1) of acyl CoA/BSA. The following acyl CoA were used: palmitoyl CoA (16:0, □), palmitoleoyl CoA (16:1n-7, ▲), oleoyl CoA (18:1n-9, ○), linoleoyl CoA (18:2n-6, ■) and docosahexaenoyl CoA (22:6n-3, ◆). See the Materials and Methods section for a full explanation of the assay system.

Since the mean $K_{0.5}$ of LF-fed animals for 16:0 is 2- to 6-fold lower than that of animals fed high-fat diets, CPT I activity is likely to saturate at a much lower endogenous concentration of palmitoyl CoA within the liver of these animals.

The decreasing order of the mean ν_{max} toward palmitoleoyl CoA (16:1n-7) was SO>MO>LF>OO (Table 2). Activity toward this acyl CoA, like that for palmitoyl CoA, was higher in SO- and MO-fed animals than in OO- and LF-fed animals (Table 2). The order of the apparent $K_{0.5}$ for palmitoleoyl CoA, which is SO>MO≈OO≈LF, revealed that the $K_{0.5}$ of the enzyme for this acyl CoA in LF-, OO-, or MO-fed animals did not differ greatly (Table 3). However, for animals fed SO, the CPT I had an approximately 2.5-fold higher $K_{0.5}$ for palmitoleoyl CoA.

TABLE 2
The Effect of Diet on the Apparent ν_{max} of Mitochondrial CPT I for Different Acyl CoA Substrates[a]

Acyl CoA	ν_{max} of CPT I ± SEM (nmol · min^{-1} · mg mitochondrial protein^{-1})			
	LF	OO	SO	MO
16:0	3.7 ± 0.2[b]	2.2 ± 0.2	4.2 ± 1.1	4.5 ± 0.7[b]
16:1n-7	30.0 ± 5.3	27.6 ± 2.6	53.9 ± 6.8[a,b]	37.7 ± 2.0[b]
18:1n-9	7.5 ± 0.3	9.5 ± 0.2[a]	12.5 ± 0.8[a,b]	22.6 ± 1.9[a,b,c]
18:2n-6	7.3 ± 0.5	8.8 ± 0.3	10.1 ± 1.5	13.0 ± 0.4[a,b]
22:6n-3	1.3 ± 0.1	2.1 ± 0.2[a]	1.8 ± 0.1[a]	3.0 ± 0.6[a]

[a]Rats were fed for 10 d on either a LF diet or one of three high-fat diets, and the ν_{max} of mitochondrial CPT I for each of five different acyl CoA substrates was determined from Lineweaver-Burk plots. Data are the mean ± SEM from three animals fed each diet. Statistical significance was determined using analysis of variance and an unpaired Student's *t*-test: a value for $P < 0.05$ was taken to indicate significance. Where they occur, significantly higher values of the ν_{max} of CPT I for individual acyl CoA substrates are indicated as follows: [a]vs. LF; [b]vs. OO; and [c]vs. SO. See the Materials and Methods section for an explanation of the diet composition, mitochondrial preparation, and assay method.

TABLE 3
The Effect of Diet on the Apparent $K_{0.5}$ of Mitochondrial CPT I Toward Different Acyl CoA Substrates[a]

Acyl CoA	$K_{0.5}$ of CPT I ± SEM (μM)			
	LF	OO	SO	MO
16:0	19.8 ± 1.9	44.2 ± 6.3[a]	114.2 ± 14.4[a,b,d]	65.2 ± 12.2[a]
16:1n-7	77.1 ± 19.1	77.6 ± 22.5	197.7 ± 24.1[a,b,d]	78.6 ± 2.7
18:1n-9	10.4 ± 1.3	14.5 ± 1.7	28.8 ± 1.7[a,b]	103.7 ± 18.9[a,b,c]
18:2n-6	31.4 ± 3.5	27.9 ± 3.4	54.9 ± 13.6	49.1 ± 2.9[a,b]
22:6n-3	8.4 ± 1.7	8.8 ± 0.8	11.2 ± 1.9	7.4 ± 2.0

[a]Rats were fed for 10 d on either a LF diet or one of three high-fat diets, and the $K_{0.5}$ of mitochondrial CPT I for each of five different acyl CoA substrates was determined from Lineweaver-Burk plots. Data are the mean ± SEM from three animals fed each diet. Statistical significance was determined using analysis of variance and an unpaired Student's *t*-test: a value for $P < 0.05$ was taken to indicate significance. Where they occur, significantly higher values of the $K_{0.5}$ of CPT I for individual acyl CoA substrates are indicated as follows: [a]vs. LF; [b]vs. OO; [c]vs. SO, and [d]vs. MO. See the Materials and Methods section for an explanation of the diet composition, mitochondrial preparation, and assay method.

The hepatic CPT I of all animals fed high-fat diets displayed a higher ν_{max} toward oleoyl CoA (18:1n-9) than LF-fed animals (Table 2). The decreasing order was MO>SO>OO>LF, with MO-fed animals exhibiting an approximately 3-fold higher mean ν_{max} than LF-fed animals. The concentration of substrate required to half-saturate the enzyme decreased in the order MO>>SO>OO≈LF (Table 3). Thus, the CPT I of animals fed either the LF or OO diet shared a similar $K_{0.5}$ for this acyl CoA. The enzyme of the SO-fed animals displayed a 2- to 3-fold higher $K_{0.5}$ for 18:1n-9, whereas that of the MO-fed animals exhibited a much higher $K_{0.5}$, being 7- to 10-fold higher.

The ν_{max} of CPT I toward linoleoyl CoA (18:2n-6) did not differ significantly between animals fed either a LF, OO, or SO diet (Table 2). Although the ν_{max} of the enzyme toward this substrate was significantly higher in animals fed the MO diet, the magnitude of the difference was small compared with other substrates such as oleoyl CoA. The apparent $K_{0.5}$ of the enzyme did not differ significantly between the LF, OO, and SO diets, whereas that of the MO-fed animals was marginally higher (Table 3).

The activity of CPT I toward docosahexaenoyl CoA (22:6n-3) was significantly higher in animals fed high fat diets than in those fed the LF diet, although generally, the ν_{max} toward this acyl CoA was relatively low for all animals (Table 2). The $K_{0.5}$ of the enzyme toward this substrate was also relatively low, with the half-saturating substrate concentration being similar in all animals (Table 3).

Thus, a consistent pattern in the ν_{max} of the enzyme for

each substrate is evident irrespective of which diet the animals were provided. The substrate for which the enzyme activity is highest is palmitoleoyl CoA, followed by oleoyl CoA, linoleoyl CoA, palmitoyl CoA, and docosahexaenoyl CoA (i.e., 16:1n-7>18:1n-9>18:2n-6>16:0 >22:6n-3). Moreover, the ν_{max} of CPT I toward all substrates was higher in animals fed either a high-fat SO or MO diet, when compared with that of animals fed either a LF or a high-fat OO diet (with only one exception, namely the ν_{max} towards 22:6n-3 of the OO- vs. SO-fed animals; Table 2). Wherever there were significant differences in the apparent $K_{0.5}$ for particular acyl CoA, the CPT I of animals fed the LF diet had a lower apparent $K_{0.5}$ than that of animals fed any of the high-fat diets (with the single exception of the $K_{0.5}$ values for 18:2n-6). Furthermore, for four of the five acyl CoA, the $K_{0.5}$ values were highest in those animals fed a high-fat SO diet (Table 3).

DISCUSSION

The range recorded in the present study of the apparent $K_{0.5}$ for palmitoyl CoA, the standard acyl CoA used in assays of CPT I, was 20–114 μM, which extends beyond the range of 30–60 μM reported by McGarry *et al.* (15), for rats fed a standard chow diet, as well as the values of 48 μM (16) and 60 μM (14) that were reported more recently. Higher values of $K_{0.5}$ for palmitoyl CoA have been reported in mitochondria from the liver of rats subjected to starvation (17,18), indicating that dietary manipulation can lead to higher $K_{0.5}$ values. It should be noted, however, that $K_{0.5}$ values from different laboratories are not strictly comparable due to differences in the albumin concentrations used in the assay, the level of which would modify the free concentration of acyl CoA available to CPT I. One should also be aware that since free CoA has been shown to inhibit CPT I activity (19), it is possible that the $K_{0.5}$ values may have been influenced by residual CoA in the acyl CoA preparations. However, this effect was probably negligible in the present investigation because free CoA is known to be highly unstable under the conditions in which the acyl CoA solutions were prepared and stored (20).

Although the data of the present investigation suggest that some fatty acyl CoA may be used preferentially by CPT I when animals are given particular diets, it is not possible to extrapolate the *in vitro* kinetic data of CPT I to the precise fatty acyl CoA partitioning that is likely to occur *in vivo*. In order to do so, it would be necessary to assess the potential influence that the diet may have on other processes that determine the free levels of acyl CoA within the liver. Thus, the relative amounts of different acyl CoA within the liver will be determined not only by the fatty acid composition of the diet but also, for example, by the extent to which individual fatty acids are absorbed, incorporated into chylomicrons, released to the peripheral tissues by lipoprotein lipase, directed to the liver as components of chylomicron "remnant" particles, and ultimately converted to acyl CoA by acyl CoA synthetase.

The decreasing order of the ν_{max} of CPT I for the different acyl CoA remained consistently as 16:1n-7>18:1n-9>18:2n-6>16:0>22:6n-3, irrespective of diet. However, the ν_{max} of CPT I toward all substrates was higher in animals fed either a high-fat SO or MO diet, when compared with those of animals fed a LF diet. It is possible that the fatty acids in these high-fat diets increased the transcription of CPT I mRNA, as has been recently reported (21), thereby increasing the enzyme concentration. However, since the increased ν_{max} were accompanied by alterations in the $K_{0.5}$ values, other mechanisms are likely to have contributed to the overall effect of these two diets. This is consistent with the observation that the OO diet generated only modest changes in the ν_{max} values, even though oleic acid, a major component of this diet, is equipotent with other long-chain fatty acids at inducing CPT I mRNA (21). It is well established that the fatty acid composition of the mitochondrial membranes is influenced by the relative contributions of the dietary fatty acids (22). CPT I interacts strongly with the outer mitochondrial membrane, and its kinetics are determined to some extent by the nature of the membrane lipids (1). It is thus possible that the diets used in the present study may have affected the biophysical properties of the outer membrane, thereby altering the conformation of CPT I resulting in a modification to its kinetic properties toward different acyl CoA.

A further possibility is that the high-fat diets may have influenced the malonyl CoA-sensitivity of CPT I, an important locus of control of this enzyme. The fact that polyunsaturated fatty acids have been found to reduce both the malonyl CoA content of liver (23) and the sensitivity of hepatic CPT I to inhibition by this ligand (4,24) may favor the oxidation of these acyl CoA when such fatty acids are consumed. A lower endogenous malonyl CoA concentration, and hence greater flux through CPT I, has been suggested by Moir *et al.* (5) to account for the decreased esterification of acyl CoA seen in liver following fish oil feeding in rats. A significantly lower sensitivity to malonyl CoA inhibition has been observed after 2 d of feeding rats on high unsaturated-fat diets similar to those used in the present investigation, when compared with animals fed either a LF diet or a high-fat diet containing a predominance of saturated fatty acids (25). The influence that the lipid components of the outer mitochondrial membrane has on the ability of malonyl CoA to transmit a conformational change to the CPT I protein is discussed elsewhere (26). It may also be possible that, since the malonyl CoA-sensitivity of CPT I appears to differ according to the type of acyl CoA utilized (27), the pattern of partitioning of acyl CoA between oxidation and esterification *in vivo* may be determined by an interplay between the composition of the acyl CoA pool and the prevailing malonyl CoA concentration.

The data show that animals given a high-fat diet demonstrate a higher CPT I activity toward the essential fatty acid 18:2n-6, when compared with animals fed a LF diet. This difference is evident in spite of the fact that the LF diet used in the present study contained amounts of 18:2n-6 considered to be adequate for normal requirements. Since CPT I in LF-fed animals possesses a comparatively high $K_{0.5}$ and a low ν_{max}

toward 18:2n-6, this may permit the sparing of this acyl CoA from β-oxidation, thus conserving this fatty acid for essential cellular processes (e.g., for the synthesis of arachidonic acid, a key component in phospholipids). Sparing of linoleic acid could also be achieved if the affinities of the mitochondrial and microsomal forms of glycerol phosphate acyltransferase, which commit acyl CoA to esterification to diacylglycerol, are comparatively higher than that of CPT I.

The oxidation of linoleate has been previously shown to be spared in animals in which the dietary availability of linoleate is compromised. Reid and Husbands (27) found that, although the CPT I specific activity was higher with linoleate and oleate than with palmitate in both sheep and rat liver, a finding consistent with the ν_{max} data for rat liver in the present investigation, the total oxidation of linoleate was lower than with the other two substrates in sheep. This "sparing" of linoleate oxidation is thought to be important in ruminant animals, because substantial amounts of this essential fatty acid are lost through the action of bacterial enzymes (biohydrogenation) in the rumen. Reid and Husbands (27) found that, in sheep, the oxidation of linoleate was inhibited at much lower concentrations of malonyl CoA than that of palmitate. It was thus concluded that the mechanism for discriminating against linoleate oxidation resides in a greater sensitivity of CPT I to malonyl CoA when utilizing linoleoyl CoA. From the results of the present investigation on rats, it is proposed that differences in the $K_{0.5}$ may provide another mechanism whereby linoleate can be conserved.

The remarkable ability of palmitoleoyl CoA to be rapidly catalyzed to its acylcarnitine by CPT I raises the possibility that this fatty acid, which is found in great abundance in the lipids of fish, may be a significant energy source for organisms whose diets contain fish oil. The dietary intake of this fatty acid may thus provide a substrate that is directed preferentially toward β-oxidation, thereby reducing the propensity to store this fatty acid as depot triacylglycerol, in situations when the dietary fat intake is not excessive. The question also arises as to why CPT I is so reactive toward this particular fatty acid. Poikilothermic animals, such as fish, contain significant amounts of long-chain polyunsaturated fatty acids (e.g., 22:6n-3) which enable their membranes to remain fluid in colder aquatic environments. However, since such fatty acids are relatively poor substrates for CPT I, there may have been strong evolutionary selection pressures for the retention, in their fat stores, of other fatty acids (e.g., 16:1n-7) that potentially offer higher rates of β-oxidation than those required for structural purposes.

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Lipid Composition of Cultured Endothelial Cells in Relation to Their Growth

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ABSTRACT: Human endothelial cells in culture were examined in different growth conditions. The human endothelial cell line, EA.hy 926 cell line, was used and cells were studied either in exponential growth phase, at confluence, or growth-arrested by serum deprivation. Phospholipids were separated and analyzed by high-performance thin-layer chromatography, and their fatty acids were quantified by gas–liquid chromatography. No significant differences in the phospholipid distributions were found between exponentially growing and confluent endothelial cells in which phosphatidylcholine (PC) represented the major phospholipid. In comparison, serum-deprived cells exhibited higher proportions of sphingomyelin and lower content of PC. We also found that among the total lipids, cholesterol level for dividing endothelial cells was lower than for cells growth-arrested either by serum deprivation or by contact inhibition at confluence. The global fatty acid distribution was not affected by the growth conditions. Thus, oleate (18:1n-9 and 18:1n-7), palmitate ($C_{16:0}$), and stearate ($C_{18:0}$) were the main components of endothelial cell membranes. However, the fatty acid distributions obtained from each phospholipid species differed with the growth status. Altogether, the data indicated that subtle modulations of endothelial cell metabolism appear upon cell growth. The resulting membrane-dependent cellular functions such as cholesterol transport and receptor activities can be expected to be relevant for lipid trafficking within the vessel wall *in vitro* and *in vivo*.
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The endothelium which lines the arterial wall is exposed to nutrients that regulate endothelial cell metabolism (1). This vascular endothelium plays an active role in physiological processes such as homeostasis, vascular permeability, and regulation of vessel tone. It is now widely recognized that cell injury that disrupts endothelium integrity is involved in pathological states such as the early events leading to the proliferation of smooth muscle cells (SMC) and the atherosclerotic lesion formation (2). The repair process in response to injury is mainly under the control of endothelial cells, tightly regulated by soluble components from the circulating blood and the surrounding cells.

In this context, phospholipids have important functions not only at a membrane level for the control of membrane fluidity and/or integrity, activity of membrane-bound enzymes, transport of molecules, and signal transduction pathways, but also at the cellular level for cell cycling, proliferation, and differentiation processes (3,4). It is likely that the growth and migration of endothelial cells alter membrane phospholipids and subsequently endothelium functions. Although phospholipid compositions of cultured endothelial cells for different species and from different origins have been reported (3,5–9), little is known about the influence of growth conditions. In an attempt to address this concern, the study described herein was designed to characterize the lipid composition of human endothelial cells as a function of the cell growth.

This work was performed with confluent, sparse serum-deprived, and exponentially growing human endothelial cells (EA.hy 926 line). We used this permanent human endothelial cell line since it maintains the characteristics of the parent human umbilical vein endothelial cells, such as the presence of factor VIII-related antigen (10), endothelin-converting enzyme activity (11), synthesis of prostaglandin I_2 and thrombomodulin (12), secretion of tissue-type plasminogen activator and plasminogen activator inhibitor (13), uptake of acetylated low density lipoprotein (LDL) (14), transport of cellular cholesterol (15), high density lipoprotein (HDL) binding (16).

MATERIALS AND METHODS

Materials. Dulbecco's minimal essential medium (DMEM), phosphate buffer saline (PBS), fetal calf serum (FCS), and L-glutamine were purchased from Gibco (Cergy Pontoise, France). Hypoxanthine–aminopterin–thymidine (HAT) and trypsin–EDTA (0.25–0.02%) were from Seromed (Berlin,

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Abbreviations: DMEM, Dulbecco's minimal essential medium; EDTA, ethylenediaminetetraacetic acid; EA.hy 926, human endothelial cell line; FCS, fetal calf serum; GLC, gas–liquid chromatography; HAT, hypoxanthine–aminopterin–thymidine; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin layer chromatography; LDL, low density lipoprotein; NL, neutral lipids; PBS, phosphate buffer saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; SMC, smooth muscle cells; TLC, thin-layer chromatography.

Germany). Solvents of high-performance liquid chromatography (HPLC) grade were from Farmitalia-Carlo Erba (Rueil-Malmaison, France). Cell culture plates from Costar (Cambridge, MA) were used. Phospholipids and fatty acids for chromatography standards were obtained from Sigma (St. Louis, MO). Silica gel (silica gel 60, 70–230 mesh) and high-performance thin-layer chromatography (HPTLC) plates (60 WRF 25 4S) were from Merck (Darmstadt, Germany).

Endothelial cell culture. Human endothelial cells (EA.hy 926 line) were kindly provided by Dr. C.-J. Edgell (University of North Carolina). They were routinely cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine and HAT (100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine) at 37°C in a 5% CO_2 incubator. Cell morphology was examined under conventional light microscope (Axiovert 100; Zeiss, Oberkochen, Germany). Absence of mycoplasma contamination was checked using the fluorescent Hoeschst 33258 dye. Protein content was determined by the Lowry method (17).

For lipid analysis, EA.hy 926 cells seeded in 162 cm^2 flasks were grown either to confluence or until the indicated density (exponential growth). In parallel experiments, some cells were growth-arrested after washing with serum-free media and incubation for 2 d in DMEM supplemented with only 0.4% FCS.

The cells were harvested using trypsin-EDTA, and the cell number was determined using a Coulter Counter (ZM type; Coultronics, Beds, United Kingdom). The doubling time (DT) (time required for the doubling of the cell population) was calculated during the exponential growth phase from the following equation:

$$DT = (t - t_0)\ \ln 2/(\ln N - \ln N_0) \quad [1]$$

where N_0 is the cell number at time t_0 and N the number at the considered time t.

Lipid analysis. Lipids were extracted by chloroform from the fixed cells according to Bligh and Dyer (18). Before thin-layer chromatography (TLC) analysis, fractionation of lipids was carried out by adsorption chromatography on silica gel in the proportion of 300 mg in chloroform for 10 mg of lipid extract. Neutral lipids were first eluted by 10 mL of chloroform. Elution of glycolipids followed with 15 mL of acetone/methanol (9:1, vol/vol), and phospholipids were recovered with 10 mL of methanol. Each fraction was evaporated, dissolved in chloroform/methanol (2:1, vol/vol), then stored at −18°C under nitrogen. Neutral lipids and phospholipids were analyzed on silica gel HPTLC plates prewashed with isopropanol and dried at 120°C for 30 min. Neutral lipid separations were performed using hexane/diethyl ether/acetic acid (70:30:1, by vol). Phospholipids were separated using HPTLC with automated multiple development. Repeated development of the plates was performed with a gradient elution, over increasing migration distances in the same dimension. The development solvent for each successive run differed, permitting a stepwise gradient to be obtained (19). All lipids were visualized by immersing plates in acetone/water (4:1, vol/vol) containing 0.5% primulin. Plates were then scanned for fluorescence at 366 nm. The CATS evaluation software package (CAMAG, Switzerland) was further used to quantify lipids. Each phospholipid class was identified by the front solvent ratio value (R_f) and compared with authentic lipid standards for calibration. R_f for phospholipids were found as follows: sphingomyelin (SM) = 0.20; phosphatidylcholine (PC) = 0.28; phosphatidylserine (PS) = 0.39; phosphatidylinositol (PI) = 0.43; phosphatidylethanolamine (PE) = 0.54.

For determination and quantification of acyl chains, total lipid extracts or lipid spots isolated from HPTLC plates were transmethylated (20). Fatty acid methyl esters of total lipids or of phospholipid species were analyzed by gas–liquid chromatography (GLC). The GC system consisted in a gas chromatograph (Autosystem Perkin Elmer, Norwalk, CT) provided with a flame-ionization detector maintained at 300°C and a programmable split/splitless injector (PSS; Perkin Elmer, Palo Alto, CA). PSS was equipped with 2-mm internal diameter liner packed with silanized glass wool. The temperature was programmed from 40°C (for 7 min) to 250°C (200°C/min) hold for 15 min. For GC separation, a 50 m × 0.22 mm internal diameter column, coated with 0.25 µm BPX 70, was used, applying helium as carrier gas. The temperature was programmed from 35°C (for 7 min) to 150°C (20°C/min), and from 150 to 230°C (1.5°C/min) hold for 5 min. Fatty acids and natural extracts of known compositions were used as standards for column calibration. Data collection was done and integrated by Turbochrom integration system (Perkin Elmer) which analyzed the weight percentage composition of the fatty acid methyl ester mixture. Values were then normalized to the total amount of identified fatty acids. The analysis procedure allows the accurate separation of double-bound position isomers. Only 18:4n-4 and 20:1n-11, which together account for less than 0.5% of the total fatty acid amount, were not resolved. Variation in surface area determination between identical injected samples was less than 2%.

Statistics. Results were expressed as mean values ± SD. The difference between two groups was tested for significance using the Student's *t*-test.

RESULTS

Cell growth. We investigated the effect of growth conditions on the lipid composition of the human endothelial cell line, EA.hy 926 (10–16,21). Three growth conditions were defined: Cells were growth-arrested by serum deprivation. They were seeded at low density ($0.8–1.2 \times 10^4$ cells/cm^2) and maintained quiescent for a two-day period in 0.4% FCS. In these culture conditions, no growth was observed. However, the cells were viable as assessed by trypan blue exclusion test, and a normal growth was recovered when serum-deprived medium was changed for 10% FCS (Fig. 1). Cells were analyzed in the exponential growth phase from day 2. In 10% FCS, these cells were actively dividing with a doubling time

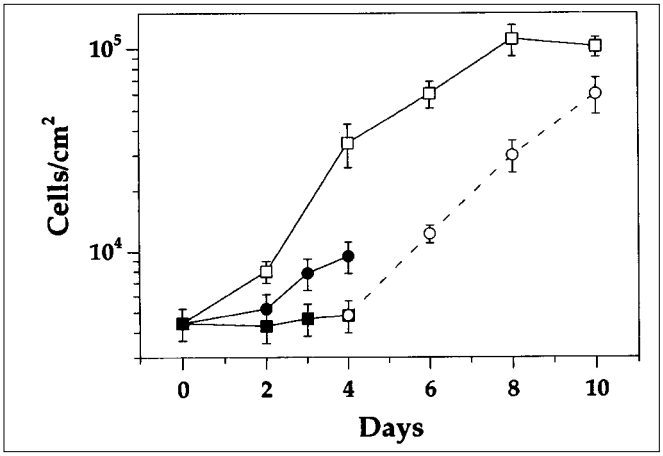

FIG. 1: Growth of human endothelial cells. EA.hy 926 cells in quadruplicate wells were seeded at 4×10^3 cells/cm^2 and cultured in DMEM supplemented with L-glutamine and HAT containing either 10% FCS (□), or 2% FCS (●). In parallel experiments, cells maintained in 0.4% FCS (■) were then allowed to grow in 10% FCS (○, dashed line). Cell numbers were determined using a Coulter counter. Mean values ± SD are indicated.

of 22.5 h. In lower serum content (2% FCS), the doubling time was on the order of 40 h. At last, confluent cells that reached a plateau at the end of the growth curve were contact-inhibited and the cell number remained constant at about 9×10^4 cells/cm^2. In this case, the typical "cobblestone" morphology of endothelial cells at confluence was observed.

Lipid composition of endothelial cells. The total lipid extracts were separated on a silica column into three species, i.e., neutral lipids (NL), glycolipids, and phospholipids. Among the total lipids, NL represented 11% of the extract, as determined by [^{14}-C]acetate incorporation (data not shown). Phospholipid compositions of EA.hy 926 cells were then determined by HPTLC with automated multiple development and densitometric scanning (Table 1). For exponentially growing cells, PC was the major phospholipid class (55.9% of total phospholipids) and smaller amounts of SM, PE, PI, and PS were found. This distribution among the individual phospholipid classes was not significantly different when compared to the one of confluent cells (Table 1). Interestingly, cells that were growth-arrested by serum deprivation differed in the phospholipid distribution, with an increase in SM and PS and a decrease in PC content.

We also investigated the cholesterol levels as a function of the growth status. With an average of 159 ± 64 µg protein/10^6 cells (n = 5), determined by the Lowry assay (17), the content of cholesterol was 40.3 ± 13.9 µg/mg cell protein for confluent cells, a value similar to those described for EA.hy 926 cells by Bernini *et al.* (16) with 32.2 µg/mg cell protein and by Kilsdonk *et al.* with 46.3 ± 5.0 µg/mg cell protein (15) and 45.3 ± 4.0 µg/mg cell protein (22). The total cellular cholesterol per cell basis (µg/10^6 cells) in the three stages of cell growth is as follows (n = 3): 1.5 ± 0.5, 6.4 ± 1.4 , and 8.7 ± 0.9 respectively for exponential, confluent and serum-deprived cells. It is worth noting that the amount of cholesterol for dividing cells is lower than for arrested cells (confluent or serum-deprived).

Fatty acid composition of endothelial cells. Table 2 shows the fatty acid distribution of total lipids recovered from endothelial cells in the three growth conditions. In confluent cells, oleate (18:1n-9; 25.6% and 18:1n-7; 6.8%), palmitate

TABLE 1
Phospholipid Composition of EA.hy 926 Cells in Different Growth Stages[a]

Phospholipids	Exponential	Confluence	Serum-deprived
PC	55.9 ± 2.4	55.1 ± 2.4	29.1 ± 1.3**
SM	20.6 ± 2.3	23.0 ± 4.1	33.5 ± 0.4*
PE	13.6 ± 0.8	14.0 ± 0.6	12.1 ± 0.7
PI	10.4 ± 2.2	7.0 ± 1.9	8.6 ± 1.8
PS	4.2 ± 0.2	3.1 ± 0.2	18.8 ± 0.8**

[a]EA.hy 926 cells grown in 162-cm^2 flasks were cultured in exponential growth phase (2.0×10^4 cells/cm^2), until confluence (7.7×10^4 cells/cm^2), or were growth-arrested by serum deprivation (1.2×10^4 cells/cm^2). The cells were harvested and total lipid extracts were separated in three species (neutral lipids, phospholipids and glycolipids). Phospholipid compositions of EA.hy 926 cells were determined by high-performance thin-layer chromatography with automated multiple development and densitometric scanning as detailed in the Materials and Methods section. Data are means ± SD of at least two separate experiments. Values are expressed as percentage of the five identified phospholipids: PC, phosphatidylcholine; SM, sphingomyelin, PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. *significantly different from exponentially growing cells ($P < 0.05$); **significantly different from exponentially growing cells ($P < 0.001$).

TABLE 2
Fatty Acid Composition of Total Lipids from Endothelial Cells[a]

Fatty acids	Exponential	Confluence	Serum-deprived
14:0	2.9 ± 0.6	2.2 ± 0.3	2.1 ± 0.2
16:0	26.8 ± 1.1	23.0 ± 2.2	25.2 ± 1.0
16:1n-9	4.3 ± 1.4	3.4 ± 0.1	3.7 ± 0.1
16:1n-7	5.8 ± 2.2	4.9 ± 0.2	4.3 ± 0.2
16:2n-6	1.1 ± 0.4	2.3 ± 0.6	n.d.
18:0	14.7 ± 1.6	14.9 ± 1.4	16.3 ± 1.6
18:1n-9	25.1 ± 1.4	25.6 ± 0.9	26.7 ± 1.5
18:1n-7	7.5 ± 0.3	6.8 ± 0.7	8.8 ± 0.6
18:2n-6	1.3 ± 0.6	2.2 ± 0.1	1.4 ± 0.1
18:3n-6	n.d.	0.3 ± 0.1	n.d.
18:4n-3	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
20:1n-9	n.d.	0.5 ± 0.1	0.7 ± 0.1
20:2n-6	0.5 ± 0.1	0.5 ± 0.1	1.0 ± 0.2
20:3n-6	0.8 ± 0.2	1.2 ± 0.1	1.7 ± 0.4
20:4n-6	3.6 ± 1.8	5.0 ± 0.2	3.4 ± 0.3
20:5n-3	0.7 ± 0.3	0.9 ± 0.1	0.8 ± 0.3
22:4n-3	0.7 ± 0.3	0.6 ± 0.1	n.d.
22:5n-3	1.6 ± 0.8	2.1 ± 0.1	1.4 ± 0.1
22:6n-6	2.3 ± 0.9	3.2 ± 0.2	2.0 ± 0.1
Saturated	44.4	40.1	43.6
Monounsaturated	42.7	41.2	44.2
Polyunsaturated	12.9	18.7	12.2

[a]EA.hy 926 cells grown in 162-cm^2 flasks were growth-arrested by serum deprivation, cultured in exponential growth phase, or until confluence. The cells were harvested and total lipids extracted as detailed in the Materials and Methods section. Data shown are expressed as percentage of the identified fatty acids and represent the average ± SD of three separate experiments. n.d., not detected.

(16:0; 23.0%), stearate (18:0; 14.9%), and palmitoleate (16:1n-7; 4.9% and 16:1n-9; 3.4%) were the main fatty acid components of endothelial cells. The other fatty acids were present in proportions lower than 7%. In all growth conditions, saturated and monounsaturated fatty acids represented more than 80% of the total molecular species. However, saturated fatty acids for confluent cells exhibited lower values (40.1%) than for the two other growth conditions (43.6% for serum-deprived cells and 44.4% for exponentially growing cells). The proportion of polyunsaturated fatty acids represented 18.7, 12.9, and 12.2%, respectively, for confluent, exponential, and serum-deprived cells. In particular, levels of 16:2n-6, 18:2n-6, 20:4n-6, and 22:6n-6 were highest in confluent cells.

The fatty acid compositions of PC, SM, PE, PI, and PS are shown in Table 3. The main fatty acids were 16:0, 16:1, 18:0, and 18:1. Each phospholipid species exhibited a unique fatty acid distribution (Table 3), in agreement with studies of others with cultured endothelial cells (7,23). For instance with confluent cells, 18:1 levels were as low as 10.9% in SM, but represented the major fatty acid component in PS with 53.4%. Culture conditions influenced the fatty acid distribution of each phospholipid class, a result not evidenced on the global fatty acid extracts. For serum-deprived cells, 18:0 was expressed at higher levels for each phospholipid class than in the two other growth conditions. Differences were observed for the fatty acid proportions in PI of exponentially growing cells (16:0/18:1 = 1.1), confluent (16:0/18:1 = 1.7), and serum-deprived cells (16:0/18:1 = 8.9). On the contrary, PC fatty acid distribution was less affected by growth conditions.

TABLE 3
Fatty Acid Composition of PC, SM, PE, PI, and PS from Endothelial Cells[a]

Phospholipids	Fatty acids	Exponential	Confluence	Serum-deprived
PC	16:0	49.1 ± 4.1	62.3 ± 7.5	52.1 ± 4.5
	16:1	11.3 ± 4.6	10.9 ± 7.3	11.3 ± 0.9
	18:0	10.1 ± 2.9	9.1 ± 4.1	15.4 ± 2.1
	18:1	29.5 ± 3.4	17.7 ± 4.3	21.2 ± 4.7
SM	16:0	55.8 ± 4.5	64.5 ± 3.3	45.4 ± 9.0
	16:1	20.2 ± 0.5	6.4 ± 3.2*	2.1 ± 2.1*
	18:0	11.1 ± 2.6	18.2 ± 6.1	38.9 ± 7.7*
	18:1	12.9 ± 2.5	10.9 ± 3.6	13.6 ± 3.7
PE	16:0	23.9 ± 1.5	33.2 ± 3.4	39.3 ± 2.3
	16:1	19.5 ± 2.5	5.9 ± 0.6*	7.4 ± 3.8
	18:0	17.3 ± 1.3	25.4 ± 3.7	33.9 ± 5.8
	18:1	39.3 ± 2.3	35.5 ± 2.7	19.4 ± 6.3
PI	16:0	37.0 ± 0.8	37.2 ± 1.3	59.5 ± 3.3*
	16:1	8.4 ± 2.9	9.1 ± 1.6	trace
	18:0	21.9 ± 1.5	32.2 ± 4.2	33.8 ± 4.3
	18:1	32.7 ± 2.2	21.5 ± 2.7	6.7 ± 0.8*
PS	16:0	42.6 ± 2.4	46.6 ± 3.3	54.4 ± 6.2
	16:1	13.4 ± 2.6	trace	8.1 ± 2.1
	18:0	16.4 ± 3.4	trace	20.8 ± 6.5
	18:1	27.6 ± 2.7	53.4 ± 3.7*	16.7 ± 3.9

[a]EA.hy 926 cells were cultured in 162-cm^2 flasks. Cells were either in the exponential growth phase (2.0×10^4 cells/cm^2), growth-arrested by serum deprivation at sparse density (1.3×10^4 cells/cm^2), or confluent (7.7×10^4 cells/cm^2). Lipids from harvested cells were extracted, and phospholipids were separated and methylated for gas–liquid chromatography analysis. Data are means ± SD of three separate experiments. Values are expressed as percentage of the predominant fatty acids (16:0, 16:1, 18:0, 18:1; see Table 2) that represented >5% of fatty acids in the total phospholipid extracts. *$P < 0.05$ vs. exponential conditions.

DISCUSSION

In physiological conditions, endothelial cells form a tightly confluent layer in the internal lumen of the vessel walls. In response to injury, proliferation of endothelial cells is a required step. Thus, atherosclerosis involves damage to or dysfunction of the vascular endothelium (2). Endothelial cell growth control is also of particular importance in wound healing and angiogenesis (24,25). Unfortunately, phospholipid compositions of cultured endothelial cells were mainly described in the literature for confluent endothelial cells (3,5–9). In order to study the regulation mechanisms of growth, we investigated the proliferation-dependent alterations in phospholipid composition of human endothelial cells in culture. We performed the analyses not only with confluent endothelial cells that would be analogous to the phenotype of *in vivo* unstimulated cells but also with exponentially growing cells and with cells whose growth was arrested by serum deprivation.

Using the HPTLC with the automated multiple development (Table 1) or a [^{14}-C]acetate incorporation (data not shown), we found that PC was the predominant species (>50%) for confluent EA.hy 926 endothelial cells. PC contents for confluent endothelial cells were similar in the literature whatever the analytical methods—TLC (5), HPLC (6), or radiolabeled tracers (8,9). PI, PE, and PS proportions determined here were also close to those previously described (5,6,8,9). Passage numbers could affect the global phospholipid composition of endothelial cells from primary culture (5,26). Lipid distribution reported in Table 1 for the human endothelial cell line EA.hy 926 was identical within 35 passages, allowing extensive investigations. The availability of this immortalized human endothelial cell line is advantageous since it increases reproducibility in longitudinal studies with an economical supply of homogeneous cellular material, unlimited replication potential, and clonal purity.

No significant differences were found for the phospholipid distribution between exponentially growing and confluent endothelial cells. However, lipid extracts obtained from serum-deprived cells exhibited higher amounts of SM and lower contents of PC. This could reflect different regulation pathways in lipid metabolism of endothelial cells. For instance, modulation of PC content by growth conditions may be relevant for the activation of protein kinase C in endothelial cells (27). On the other hand, the phospholipase C activity devoted to second-messenger production was not significantly altered by PC, while SM inhibited all phospholipase C activities (28). The enrichment of SM in serum-deprived endothelial cells may have physiological significance (29). Indeed, SM and its hydrolysis product by sphingomyelinase into ceramide participate in important cellular processes such as differentiation,

proliferation, and apoptosis (30,31). Modulation of cell growth by physical factors such as shear stress or by addition of growth-regulating compounds (25,32–36) that mediated lipid metabolism remains to be explored in great detail with cultured endothelial cells. These precise cellular and molecular regulatory processes in relation to cell growth are likely to play a significant role both *in vitro* and *in vivo*.

Lipoprotein uptake by vascular endothelial cells has been implicated in the initiation and development of atherosclerosis. Cholesterol trafficking may contribute to HDL and oxidized LDL binding in atherogenesis. HDL is believed to play a key role in the transfer of excess cellular cholesterol *via* an interaction of HDL with a membrane receptor (15,37). It was also shown that EA.hy 926 cells represent a valid model for studying the *in vitro* interaction of HDL with human endothelial cells (16). For these cells, cholesterol enrichment participates in the upregulation of HDL binding (15,16). Low cellular cholesterol levels were found in exponentially growing EA.hy 926 cells. This differential regulation may be indicative of regulation processes occurring at the LDL receptor level. This is important since LDL receptor and the scavenger receptor are the two main cell surface receptors by which vascular cells and macrophages bind and internalize LDL cholesteryl ester and deliver cholesterol to the cells.

We then investigated the fatty acid distribution of EA.hy 926 cells in different growth conditions (Table 2). Again, the global fatty acid distributions are only described in the literature for confluent endothelial cells. The levels of fatty acids obtained with confluent EA.hy 926 cells were similar to those described for other confluent endothelial cells (7,22,38). When we compared the three growth conditions, we observed that confluent cells exhibited higher amounts of polyunsaturated fatty acids than the two other growth stages. For instance, arachidonic acid in confluent cells reached 5% of the total fatty acids, whereas this level was only 3.5% for the other growth conditions. In fact, arachidonic acid metabolism is described as a key factor for the regulation of the endothelial cell proliferation (26). It is also interesting to note that growth-arrested cells obtained either by contact inhibition of confluent monolayers or by serum deprivation differed in their content of polyunsaturated fatty acid. This result evidences that macroscopic observation of nonproliferation is, at least partially, controlled by lipid metabolism. Fatty acid distributions were also obtained here for each individual phospholipid. These distributions were already described with rabbit coronary microvessel endothelial cells (7) and arteries and veins from human umbilical cords (5), but to our knowledge this is the first report of fatty acid distributions in each phospholipid from the human endothelial cell line, EA.hy 926. The results showed that different distributions were obtained among PC, SM, PE, PI, and PS (Table 3). Substantial changes were found as a function of growth. The differences pointed out both in the fatty acid distribution for a given phospholipid, and in the content for one fatty acid among the five analyzed phospholipids. Thus, the specific phospholipid composition related to membrane fluidity and integrity is dependent on the cell growth stage. The resulting alterations of human endothelial cell membranes are relevant for pharmaceutical treatments and should be of interest in the understanding of the pathophysiology of the vascular walls.

In conclusion, it is important to take into account the complexities of phospholipid metabolism in living cells when studying the effects of parameters that could affect the cell behavior. Their modulating role in endothelial cells is relevant for lipid trafficking within the vessel wall. Our results indicated that the lipid composition of the permanent human endothelial cell line, EA.hy 926, was in good agreement with the compositions described for primary cultures of endothelial cells. Moreover, the importance of growth status on lipids extracted from endothelial cells has been underlined. This work will support further investigations in the control of growth for endothelial and smooth muscle cells from the vascular wall submitted to physical and chemical stresses.

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Effect of Radiotherapy and Chemotherapy on Composition of Tumor Membrane Phospholipids

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ABSTRACT: Phospholipid extracts were made of a murine mammary adenocarcinoma implanted in the dorsum of the foot of C3H/He mice before and 96 h after 17 Gy irradiation or 150 mg/kg cyclophosphamide. Extracts of untreated tumors, which had grown for a further 96 h, were also studied. Although previous studies have shown significant changes in the precursors and catabolites of phosphatidylcholine and phosphatidylethanolamine following therapy, ^{31}P nuclear magnetic resonance analysis of extracts showed no changes in these membrane constituents and other observed phospholipid species. A significant decrease in 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine, however, was observed 96 h following cyclophosphamide treatment. *Lipids 32,* 45–49 (1997).

It has been well documented that the precursors of phospholipids, the phosphomonoesters, change significantly in tumors in response to different therapies (1–5). In previous studies we have shown an increase in the ratio downfield component of the phosphomonoester region of ^{31}P nuclear magnetic resonance spectrum, comprised of primarily PEth and nucleoside monophosphates (PME′)/phosphocholine (*P*Cho) following both radiotherapy *in vivo* (6,7) and *in vitro* (8) and following cyclophosphamide chemotherapy *in vivo* (9). PME′ was defined in these studies as the downfield phosphomonoester peak, comprised principally of phosphoethanolamine (*P*Etn) and nucleoside monophosphates. Other studies have shown similar increases in *P*Etn/*P*Cho following chemotherapy (10,11). Similar increases in phospholipid catabolites, including the phosphodiesters, have been noted post therapy (6–9,12); other reports, however, have noted a decrease (13,14).

The phospholipids themselves are constituents of cell membranes and, as such, are relatively immobile molecules. This immobility causes spin-spin relaxation times (T_2) to be very short causing resonances in the nuclear magnetic resonance (NMR) spectrum to be very broad *in situ*. This means that changes in the composition of the phospholipid membrane are less well understood than those of their metabolites. In order to investigate the composition of tumor membrane phospholipids, it is necessary to make lipid extracts of cells and tissues, so that they can be studied in solution, where the T_2 are much longer. Tumor spheroids, which showed changes in phosphomonoester levels with increasing size, were found to have a tightly regulated phosphatidylethanolamine/phosphatidylcholine (PtdEtn/PtdCho) ratio (15). Merchant *et al.* (16), however, observed an increase in PtdEtn/PtdCho following high doses of radiation *in vivo*, correlating with an increase in *P*Etn/*P*Cho. The objective of this study was to further investigate any changes in membrane phospholipid composition following either radiotherapy or chemotherapy in a mouse tumor model.

METHODS

Animals. MCa tumors, a mouse mammary adenocarcinoma tumor (17), were grown on the dorsum of the foot of male C3H/He mice (Jackson Laboratory, Bar Harbor, ME) as described previously (6). Tumors of 245 ± 24 mm^3 volume were either treated with 17 Gy irradiation, 150 mg/kg cyclophosphamide, or were untreated. Treated animals and one group of untreated animals were sacrificed at 96 h post treatment; a second untreated group were sacrificed at zero hours. Each group consisted of six or eight animals. Two tumors were combined for each extract.

Extraction. Tumors were excised by incising at right angles to the dorsal surface of the skin adjacent to the tumor and then were freeze clamped by immediately dropping into liquid nitrogen. This freeze-clamping technique, for tumors of this size, has been shown to be not significantly different from freezing between liquid nitrogen-cooled aluminum blocks (18). The tumor tissue was then ground to a powder in a liquid nitrogen-cooled mortar. Chloroform/methanol extracts were then made as described by Meneses and Glonek (19).

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Abbreviations: ANOVA, analysis of variance; Cer*P*Cho, sphingomyelin; LPC, lysophosphatidylcholine; NMR, nuclear magnetic resonance; PA, phosphatidic acid; *P*Cho, phosphocholine; *P*Eth, phosphoethanolamine; PlaCho, 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; PlsEtn, ethanolamine plasmalogen; PME′, downfield component of the phosphomonoester region of ^{31}P nuclear magnetic resonance spectrum, comprising primarily *P*Eth and nucleoside monophosphates; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; Ptd_2Gro, cardiolipin; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine.

The pulverized tissue was vortexed for 2 min with 20 vol of chloroform/methanol (2:1, vol/vol). This was then washed with 4 tumor volumes of KCl (0.1 M) and vortexed for a further 1 min. This emulsion was allowed to separate overnight. The lower chloroform phase was removed and evaporated to dryness by passing a stream of air over it. The resulting lipid residue was reconstituted in 1 mL deuterated chloroform and 0.5 mL methanol:0.2 M Cs-EDTA, pH 6.0 (4:1, vol/vol).

NMR. Gated proton decoupled ^{31}P NMR spectra of extracts were obtained on a Bruker AC 250 MHz spectrometer (Karlsruhe, Germany) using a 5-mm quad probe operating at 101.25 MHz, as described by Meneses and Glonek (19). The spectral parameters used were a 45° pulse width (2.8 µs), acquisition delay of 500 µs, relaxation delay of 500 ms, 2000 Hz spectral width, and 4k data points (1.02 s acquisition time). Transients (2048) were accumulated. Relative saturation effects were not detectable under these conditions (19). Spectra were referenced to trimethyl phosphate at 0 ppm, and peak assignments were made by comparison of spectra to previously reported data using similar techniques (16,20). The signal-to-noise ratio for PtdCho was typically 45:1 for a spectrum of a pre-treatment specimen, and better in spectra of larger tumors. For quantitation of peak areas, spectra were zero-filled and processed by Fourier transform and estimated by integration. Under these conditions all of the spectral components quantified were fully resolved. The spectrum shown in Figure 1 was transformed with 0.6 Hz exponential line-broadening for display purposes. Differences were analyzed using analysis of variance and least squared means tests. Error limits are ± 1 SD of the mean, except where stated.

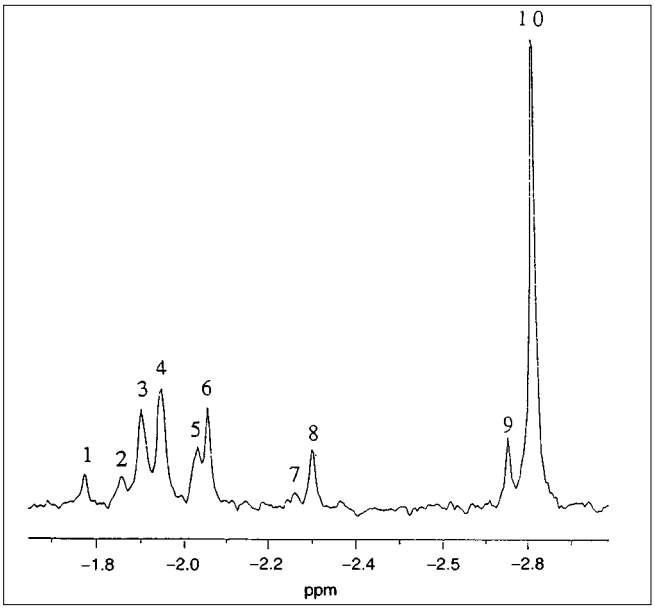

FIG. 1. ^{31}P nuclear magnetic resonance spectrum of phospholipid extract of untreated MCa tumor at zero hours. Peak assignments are as follows: 1) phosphatidic acid; 2) cardiolipin; 3) ethanolamine plasmalogen; 4) phosphatidylethanolamine; 5) phosphatidylserine; 6) sphingomyelin; 7) lysophosphatidylcholine; 8) phosphatidylinositol; 9) 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; and 10) phosphatidylcholine.

RESULTS

Previous studies with the MCa tumor model (7,9) have shown significant growth delay following 17 Gy irradiation (n = 16), 150 mg/kg cyclophosphamide (n = 8) compared to untreated tumors (n = 7) (both $P < 0.05$ after 96 h). The mean volumes (±1 SD) of the treated tumors used for the phospholipid extracts, at the time of extraction, were 337 ± 51 mm^3 for 17 Gy treated tumors and 410 ± 63 mm^3 for cyclophosphamide treated tumors. The extracted untreated tumors had a volume of 245 ± 24 mm^3 at zero hours and 632 ± 263 mm^3 after 96 h. The tumor growth delay to reach double the volume for these therapies was 3 d and 2 d following 17 Gy irradiation and 150 mg/kg cyclophosphamide, respectively. Post therapy (96 h) was chosen for harvesting of tumors for lipid extraction, as this time point yielded the greatest changes in phospholipid metabolites *in vivo* (7,9).

Figure 1 shows a typical ^{31}P NMR spectrum of a phospholipid extract of an untreated tumor. Addition of Cs-EDTA as a chelating agent in the final step of the extraction protocol caused significant narrowing of line widths (19), allowing ten phospholipid species to be identified. This improved spectral resolution has allowed us to investigate changes in the phospholipid composition of tumors upon growth and following therapy. Sphingomyelin (Cer*P*Cho) and phosphatidylserine (PtdSer) could not be resolved sufficiently in most of the spectra for purposes of quantitation. The peak at −2.75 ppm in Figure 1, 0.06 ppm downfield of PtdCho, has been assigned by some workers to choline plasmalogen (19,21) and 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (PlaCho) by others (22–24). Comparison of the tumor extract spectrum with that of beef heart PtdCho (Sigma, St Louis, MO), which contains approximately 30% plasmalogens and a small quantity of PlaCho, indicated that the peak observed in the tumor spectrum was that of PlaCho rather than the plasmalogen. The plasmalogen peak was separated from the PtdCho peak by approximately 0.02 ppm. These assignments are in agreement with Edzes *et al.* (23) who identified this peak, using 2D NMR and saponification techniques. These workers observed not only PlaCho but also choline plasmalogen at a different chemical shift, closer to PtdCho.

Absolute quantitation of metabolites dissolved in chloroform is very difficult owing to the inaccuracies associated with dispensing small volumes of a volatile organic solvent with a low surface tension. This has necessitated the use of phospholipid ratios (16) or mole percentages (22,23), rather than absolute values of concentration, for purposes of quantitation in most previous studies. Table 1 shows the tumor content of phosphatidic acid (PA), cardiolipin (Ptd_2Gro), lysophosphatidylcholine (LPC), and phosphatidylinositol (PtdIns) relative to PtdCho under each of the conditions studied. It can be seen that each of these ratios was invariant both with tumor growth and following both 17 Gy irradiation and

TABLE 1
^{31}P Nuclear Magnetic Resonance Phospholipid Ratios of Minor Phospholipid Components, Relative to Phosphatidylcholine (PtdCho)[a]

	Control (0 h)	Control (96 h)	Cyclophosphamide (150 mg/kg)	Irradiation (17 Gy)
PA/PtdCho	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.04	0.05 ± 0.01
Ptd_2Gro/PtdCho	0.06 ± 0.02	0.10 ± 0.07	0.07 ± 0.03	0.04 ± 0.01
LPC/PtdCho	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
PtdIns/PtdCho	0.10 ± 0.03	0.09 ± 0.03	0.12 ± 0.04	0.11 ± 0.02

[a]Errors are ±1 SD. PA, phosphatidic acid; Ptd_2Gro, cardiolipin; LPC, lysophosphatidylcholine; PtdIns, phosphatidylinositol.

150 mg/kg cyclophosphamide. Figure 2A shows the ratios of PtdEtn/PtdCho and PlsEtn/PlaCho under the four conditions studied. The ratio PtdEtn/PtdCho was 0.31 ± 0.03, 0.31 ± 0.01, 0.28 ± 0.05 and 0.31 ± 0.05 for control tumors at zero hours, control tumors at 96 h, tumors treated with cyclophosphamide and 17 Gy irradiated tumors, respectively. None of these ratios are significantly different from each other. ANOVA, however, indicated that the various conditions resulted in signifi-cant differences in PlsEtn/PlaCho (F = 12.5; P = 0.001). PlsEtn/PlaCho following cyclophosphamide treatment was significantly higher than both control groups and following irradiation, as determined by least squared means ($P < 0.001$). PlsEtn/PlaCho was 3.29 ± 0.60 in cyclophosphamide treated animals, 1.97 ± 0.16 in controls at 96 h and 1.98 ± 0.18 in controls at 0 h. This ratio in 17 Gy irradiated tumors was 2.04 ± 0.18. Figure 2B shows the ratios PlsEtn/PtdEtn and PlaCho/PtdCho. There was no significant change in the ratio PlsEtn/PtdEtn upon growth of tumors or following therapy. PlsEtn/PtdEtn for control tumors at 0 h and untreated tumors after 96 h were 0.79 ± 0.10 and 0.67 ± 0.06, respectively. Following cyclophosphamide therapy and radiotherapy, PlsEtn/PtdEtn was 0.82 ± 0.12 and 0.66 ± 0.04, respectively. PlaCho/PtdCho was 0.12 ± 0.02 for controls at 0 h, 0.11 ± 0.01 for untreated tumors after 96 h, 0.07 ± 0.02 for cyclophosphamide treated tumors, and 0.10 ± 0.01 for 17 Gy irradiated tumors. ANOVA indicated significant differences in PlaCho/PtdCho between the different groups (F = 7.5; P = 0.006). Least squared means analysis indicated that PlaCho/PtdCho was significantly lower than both control groups ($P < 0.003$) but was not significantly different from irradiated tumors. Also, there was no significant change in this ratio upon growth of the tumor or following radiotherapy.

DISCUSSION

The data presented here indicate that there was no change in the ratio PtdEtn/PtdCho, although our previous studies with this tumor model have shown significant alterations in the phosphomonoesters and phosphodiesters, intermediates of the biosynthetic and degradative pathways of phospholipids, respectively, following radiation and cyclophosphamide therapy (7–9). These results suggest that levels of these major constituents of the tumor membrane are tightly regulated, despite apparent changes in their metabolism. This is in agreement with Ronen *et al.* (15) who observed no change in PtdEtn/PtdCho upon growth of T47D human breast cancer

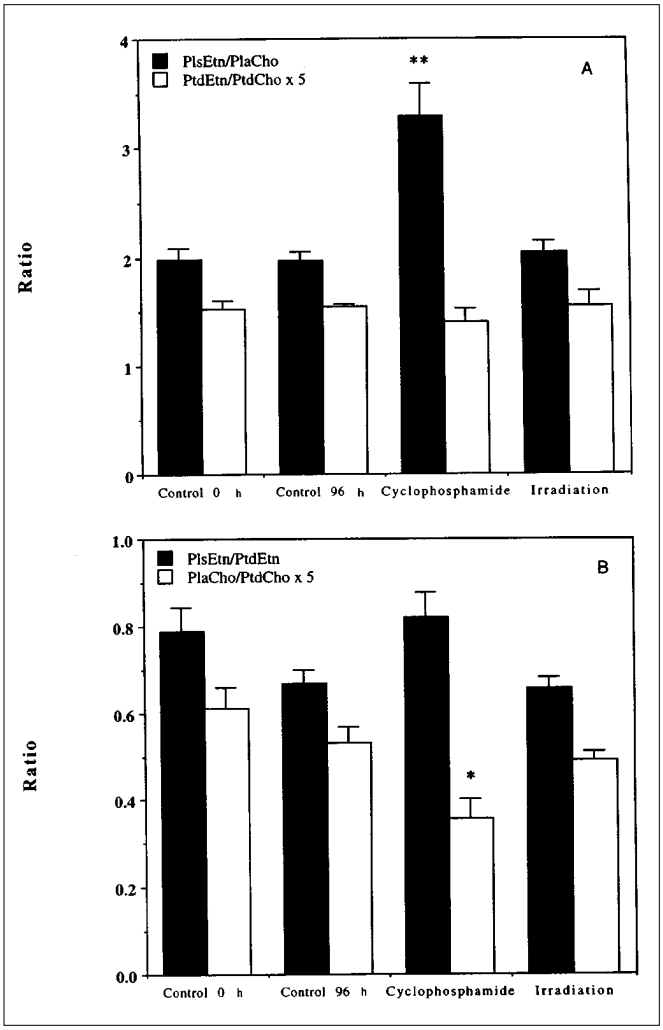

FIG. 2. Chart showing phospholipid ratios observed in ^{31}P nuclear magnetic resonance spectra of extracts of MCa tumors. A) ethanolamine plasmalogen/1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (PlsEtn/PlaCho) and phosphatidylethanolamine/phosphatidylcholine (PtdEtn/PtdCho) and B) PlsEtn/PtdEtn and PlaCho/PtdCho. PtdEtn/PtdCho and PlaCho/PtdCho have been multiplied by a factor of five for ease of comparison. Error bars are standard error of the mean. *Significant differences relative to controls of $P < 0.003$. **A difference relative to controls of $P < 0.001$.

spheroids, despite a significant change in *P*Etn/*P*Cho. Merchant *et al.* (16), however, reported an increase in PtdEtn/PtdCho following radiotherapy in MCa tumors. The doses of irradiation, 32 and 65 Gy, used in that study were much higher than that used here and may have caused extensive cell damage including direct damage to cellular proteins, inhibition of glycolysis, and membrane damage mediated by free radicals as well as effects on DNA and RNA (25). In addition to PtdEtn and PtdCho, there was no change in the membrane composition of a number of other phospholipid species, including PA, Ptd_2Gro, LPC, PtdIns, and PlsEtn. It is well documented that changes occur in both phosphomonoesters and phosphodiesters following therapy (26), suggesting that any membrane changes in these metabolites following therapy are due to an effect on membrane turnover rather than a direct effect of therapy on membrane composition.

The only significant changes observed in the phospholipid profile of tumors in response to therapy in this study were a decrease in PlaCho/PtdCho and an increase in PlsEtn/PlaCho following cyclophosphamide. Given that no other ratios were affected, this indicates a reduction in PlaCho. No change was observed following 17 Gy irradiation, or in untreated tumors, suggesting that this may be a cyclophosphamide-specific change, although a similar response at higher doses of radiation cannot be ruled out. In fact Merchant *et al.* (16) did observe an increase in PlaCho at higher doses of radiation. Although the function of plasmalogens and alkylacyl glycerophospholipids remain obscure, diradylglycerols, metabolites of plasmalogens and alkylacyl glycerophospholipids, have been shown to activate protein kinase C (27), and PlaCho is known to be a precursor of platelet-activating factor (28). Elevated levels of PlaCho have been correlated with metastatic potential in rat mammary carcinomas (29) and in clones of T3 cells of different metastatic potential (30). It is possible therefore that the reduction in PlaCho following cyclophosphamide therapy in this study is related to a loss in the metastatic potential of the tumor.

In this study we found that levels of the major phospholipid components of tumor cell membranes, PtdEtn and PtdCho, were not affected by antitumor therapies which have previously been shown to have significant effects on their anabolites and catabolites. This suggests that these changes in metabolism reflect a change in phospholipid turnover, i.e., a change in the rate of synthesis and breakdown of phospholipid following arrest of tumor growth, rather than a gross change in the composition of the membrane itself. In addition we have observed a decreased proportion of PlaCho following cyclophosphamide.

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Dietary Very Long Chain Fatty Acids Directly Influence the Ratio of Tetracosenoic (24:1) to Tetracosanoic (24:0) Acids of Sphingomyelin in Rat Liver

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ABSTRACT: Twenty-one groups of weanling male Wistar rats were fed semipurified diets containing 5% (w/w) of different dietary fats. After 2 wk, liver sphingomyelin (SM) fatty acid composition was determined. The ratio of 24:1 to 24:0 in liver SM varied over a tenfold range in response to dietary fat type. Stepwise multiple regression analysis indicated that dietary 24:1, 24:0, and 22:1 were the most significant factors in predicting the 24:1/24:0 ratio of liver SM. The mathematical relation between the dietary fatty acid composition and liver SM 24:1/24:0 was $y = 1.88$ (24:1) -1.49 (24:0) + 0.21 (22:1) + 0.01 (18:1) + 0.26, $r^2 = 0.95$, $P < 0.0001$. These results were confirmed by a second experiment in which the rats were fed olive oil-based diets supplemented with various fatty acid ethyl esters.
Lipids 32, 51–55 (1997).

Dietary fat type has been shown to alter the fatty acid composition of tissue sphingomyelin (SM) in various species (1–7): however, the relationships between the intake of specific fatty acids and the changes in tissue SM fatty acid composition have not been defined. Recently, we reported that rats fed diets containing 5% herring oil had over fourfold higher level of the 24:1/24:0 ratio in SM in liver and heart compared with rats fed diets containing 5% corn oil (8). The present experiments were designed to investigate the relationship between the intake of specific dietary fatty acids and the 24:1/24:0 ratio in rat liver SM.

MATERIALS AND METHODS

Male weanling Wistar rats (45–50 g) were fed, for 14 d, a semipurified diet consisting of vitamin-free casein (20%), DL-methionine (0.3%), glucose hydrate (65%), fat (5%), cellulose (5%), choline bitartrate (0.2%), vitamins and minerals as previously described (9), but providing 30 mg Zn/kg diet. The rats were housed under controlled conditions of temperature (22°C), humidity (50%), and lighting (12 h day/night) and were provided with food and distilled water *ad libitum*.

In experiment 1, the rats were fed the experimental diets with a variable fat source ($n = 3$, 21 diets). Menhaden oil was obtained from Sigma Chemical (St. Louis, MO). Borage and Lorenzo's oils were obtained from Dr. B.J. Holub (University of Guelph) and herring oil from Dr. C.Y. Cho (University of Guelph). All other fats and oils were obtained from local supermarkets and health food stores. Egg yolk fat was fed as whole egg yolk from hard-boiled eggs. The dietary fats were transesterified and the fatty acid methyl esters analyzed by gas–liquid chromatography as previously described (10); fatty acid profiles are shown in Table 1. In experiment 2, the rats ($n = 3$) were fed a diet of 5% olive oil plus the ethyl esters of 20:0, 20:1 (*cis*-11-eicosanoic acid), 22:0, 22:1 (*cis*-13-docosenoic acid), 24:0, or 24:1 (*cis*-15-tetracosenoic acid) (Sigma Chemical) at a level of 2.0 wt% of the oil (0.1 wt% of the total diet). The analytical methods for SM extraction, purification, and analysis of fatty acid methyl esters were previously described (10).

Data from the experiments were analyzed by general linear modeling with multiple means comparisons using Tukey's test. Linear regression of each fatty acid in the dietary oil with the corresponding fatty acid in SM was performed. Stepwise multiple regression between dietary fatty acids and the 24:1/24:0 ratio in SM was performed on the data from experiments 1 and 2.

RESULTS

At the end of the two-week feeding trials, all rats appeared healthy. Body weight gain was not significantly different between the groups of rats fed the experimental diets.

The effects of different dietary oils on the fatty acid composition of rat liver sphingomyelin are shown in Table 2. There was significant variation of SM fatty acid composition between groups of rats fed diets containing the 21 test oils. Specifically, there was significant variation (Tukey's test, $P < 0.05$) in 18:0, 18:1, 20:0, 22:1, 23:0, iso 24:0, 24:0, 24:1, and 24:2 of SM. A regression analysis between fatty acids in the diet with the corresponding fatty acid in SM demonstrated that there was a significant positive correlation of dietary 24:1 with SM 24:1 ($r^2 = 0.35$, $P < 0.0001$), dietary 22:1 with SM 22:1 ($r^2 = 0.38$, $P < 0.0001$), and dietary 22:0 with SM 22:0

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Abbreviations: PUFA, polyunsaturated fatty acids, SM, sphingomyelin.

TABLE 1
Fatty Acid Composition of Dietary Oils[a]

Fatty acid	Lor	Bor	Her	Cod	Shr	Can	Men	Lard	Alm	Egg	But
12:0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.65
14:0	0.05	ND	2.96	1.61	0.33	0.05	12.03	1.79	ND	0.52	14.77
14:1	ND	ND	7.12	4.17	ND	ND	ND	ND	ND	ND	1.54
16:0	0.78	11.03	12.79	11.49	13.64	6.36	22.34	29.62	7.75	31.07	35.46
16:1	ND	ND	12.36	13.00	ND	ND	15.58	3.42	ND	3.85	1.10
18:0	1.99	3.51	0.99	2.33	11.26	1.85	3.58	15.20	0.94	8.41	12.30
18:1	77.97	16.84	9.94	26.20	62.71	65.95	13.69	40.81	63.88	42.77	25.33
18:2	4.65	34.26	1.24	4.00	9.53	20.70	1.27	6.76	27.22	11.59	2.14
18:3n6	ND	27.09	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:3n3	ND	ND	0.18	1.43	0.62	1.80	1.61	0.73	0.00	0.45	0.73
20:4n6	ND	ND	0.28	0.29	ND	ND	0.62	0.11	ND	1.27	0.18
20:5n3	ND	ND	4.73	7.73	0.04	ND	10.98	0.01	ND	0.01	0.08
22:6n3	ND	ND	0.42	5.35	ND	ND	7.79	0.01	ND	0.43	ND
20:0	0.55	0.18	0.16	0.06	0.54	0.74	0.16	0.23	ND	0.07	0.20
20:1	0.26	3.67	18.23	10.30	0.86	1.51	1.58	0.74	0.09	0.22	0.30
22:0	0.01	0.13	0.02	0.94	0.13	0.29	0.09	0.01	0.07	0.02	0.06
22:1	13.60	2.10	23.17	5.80	0.18	0.55	0.19	0.02	ND	0.02	0.02
24:0	ND	0.02	2.31	0.23	0.04	0.08	0.07	ND	ND	ND	0.01
24:1	0.14	1.03	0.66	0.27	0.04	0.11	0.24	ND	ND	0.01	ND
PUFA	4.65	61.35	6.85	18.80	10.19	22.50	22.29	7.62	27.22	13.74	3.14
MUFA	91.97	23.63	71.48	59.74	63.79	68.13	31.28	44.99	63.97	46.88	28.29
SAT	3.36	14.86	19.23	16.66	25.93	9.37	38.26	46.85	59.97	40.09	66.45

Fatty acid	Oli	Cbt	Saff	Coc	Corn	Soy	Flax	Prim	Pea	Sun
12:0	ND	ND	ND	54.17	ND	ND	ND	ND	ND	ND
14:0	ND	0.11	0.13	24.91	0.02	ND	0.10	0.04	0.04	0.05
14:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
16:0	18.10	29.11	8.06	11.06	12.50	12.78	8.35	8.17	13.02	7.99
16:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:0	1.90	32.85	2.09	2.76	1.35	4.00	4.04	1.64	2.65	5.20
18:1	59.97	33.63	16.46	5.15	30.99	24.42	16.09	9.22	51.60	16.52
18:2	18.63	3.00	72.83	1.12	54.42	52.88	19.52	69.71	26.97	69.45
18:3n6	ND	ND	ND	ND	ND	ND	ND	9.96	ND	ND
18:3n3	ND	ND	ND	ND	0.12	2.71	50.18	ND	0.06	0.04
20:4n6	ND	ND	ND	0.10	ND	0.04	ND	ND	ND	ND
20:5n3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
22:6n3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
20:0	0.28	0.90	0.18	0.16	0.37	0.36	0.11	0.32	1.32	0.41
20:1	0.20	0.07	0.11	0.15	0.20	0.16	0.08	0.14	1.16	0.12
22:0	ND	0.18	0.04	0.08	0.02	0.37	0.03	0.07	2.25	0.20
22:1	ND	ND	ND	ND	ND	ND	ND	ND	0.06	ND
24:0	ND	0.05	ND	ND	ND	0.05	ND	0.01	0.78	0.01
24:1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PUFA	18.63	3.00	72.83	1.22	54.53	55.62	69.70	79.67	27.04	69.49
MUFA	60.17	33.70	16.58	5.30	31.19	24.58	16.17	9.36	52.82	16.64
SAT	20.28	63.20	10.50	93.14	14.26	17.56	12.63	10.25	20.07	13.86

[a]Values are means of n = 3, expressed as mol%. ND, <0.005 mol%. PUFA, polyunsaturated fatty acids; MUFA monounsaturated fatty acids; SAT, saturated fatty acids. Alm, almond oil; Bor, borage oil; But, butter; Can, canola oil; Cbt, cocoa butter; Coc, coconut oil; Cod, cod liver oil; Her, herring oil; Lor, Lorenzo's oil; Men, menhaden oil; Oli, olive oil; Pea, peanut oil; Prim, evening primrose oil; Saff, safflower oil, Shr, vegetable oil shortening; Soy, soybean oil.

($r^2 = 0.23$, $P < 0.0001$). A regression analysis between dietary polyunsaturated fatty acids (PUFA), monounsaturated fatty acids and saturated fatty acids, and the 24:1/24:0 ratio in SM indicated that there was a significant positive correlation of dietary monounsaturated fatty acids ($r^2 = 0.34$, $P < 0.0001$) and significant negative correlations of dietary saturated fatty acids ($r^2 = 0.07$, $P < 0.05$) and PUFA ($r^2 = 0.08$, $P < 0.02$), with the 24:1/24:0 ratio in SM. Stepwise multiple linear regression between the fatty acid composition of the diet (Table 1) and the 24:1/24:0 ratio in SM (Table 2) indicated that 24:1, 24:0, 22:1, and 18:1 had the greatest effects; the regression formula was $y = 1.88\ (24:1) - 1.49\ (24:0) + 0.21\ (22:1) + 0.01\ (18:1) + 0.26$, $r^2 = 0.95$, $P < 0.0001$ (Table 3). The concentration unit of the fatty acids in the regression formula is expressed as mole percentage of total fatty acids in the dietary fat, based on assay diets containing 5% added fat. The plot of predicted versus observed effects is shown in Figure 1.

The effects of different fatty acid ethyl esters supplemented in the diets of rats on liver SM fatty acid composition are shown in Table 4. The feeding of fatty acid ethyl ester-

TABLE 2
Sphingomyelin Fatty Acid Composition of Rat Livers[a]

Fatty acid	Lor	Bor	Her	Cod	Shr	Can	Men	Lard	Alm	Egg	But
16:0	20.70	21.55	22.33	23.43	23.59	17.92	27.16	17.87	25.54	23.73	21.63
18:0	5.24	14.48	6.48	5.67	9.40	4.76	6.80	7.92	7.33	9.13	9.60
18:1	1.69	6.37	0.89	0.34	ND	ND	0.81	0.24	ND	0.66	0.76
18:2	0.23	2.56	1.30	0.48	0.06	0.06	ND	0.05	0.31	0.24	0.16
20:0	1.06	1.79	1.41	2.10	2.60	2.12	1.97	2.41	1.86	2.41	1.74
22:0	3.21	4.81	5.81	8.74	10.72	11.20	9.11	11.16	7.36	9.63	10.08
22:1	4.80	0.47	1.70	0.78	0.58	0.30	0.87	0.45	0.20	1.26	1.44
23:0	3.50	2.75	3.90	5.58	6.59	5.60	6.62	6.85	7.32	6.30	7.80
iso 24:0	2.13	1.26	2.74	2.23	2.41	3.23	2.83	2.92	4.21	4.36	3.77
24:0	10.03	11.06	13.44	17.69	21.16	22.88	21.99	24.87	22.99	21.64	22.55
24:1	46.49	31.69	37.97	30.94	21.10	25.48	19.74	21.86	19.42	17.99	17.95
24:2	1.55	1.23	2.02	1.97	1.79	2.33	2.09	3.40	3.45	2.60	2.52
Unidentified	7.45	5.58	5.72	ND	2.06	5.43	5.20	10.47	5.80	6.79	5.97
24:0 + 24:1	56.52	42.75	51.41	48.62	42.26	52.37	41.74	46.74	42.41	39.64	40.50
24:1/24:0	4.63	2.93	2.83	1.78	1.00	0.95	0.90	0.88	0.85	0.83	0.79

Fatty acid	Oli	Cbt	Saff	Coc	Corn	Soy	Flax	Prim	Pea	Sun
16:0	20.88	24.86	21.58	24.51	18.49	20.16	18.04	27.55	17.31	18.37
18:0	10.05	11.59	9.18	7.89	5.40	6.17	8.30	7.30	5.74	6.11
18:1	ND	0.68	ND	4.31	ND	ND	2.23	ND	ND	0.37
18:2	0.44	0.21	0.34	1.85	0.23	0.18	2.19	ND	0.15	0.19
20:0	2.84	3.43	2.14	1.83	2.43	2.13	4.16	2.62	1.94	2.13
22:0	11.18	11.75	12.12	8.30	11.35	12.15	11.84	12.08	18.20	17.05
22:1	ND	0.29	0.56	0.72	0.14	0.20	1.78	0.85	0.49	0.33
23:0	7.99	6.11	6.82	6.33	7.92	8.52	6.87	7.27	4.56	6.77
iso 24:0	3.28	2.57	3.38	4.19	5.00	4.33	3.31	3.44	2.18	3.71
24:0	24.20	22.17	26.58	25.25	31.28	29.13	28.21	26.23	37.17	31.56
24:1	16.93	14.43	13.34	11.85	13.31	12.28	11.09	9.36	10.77	9.08
24:2	1.87	1.40	3.96	2.57	4.46	4.76	1.99	3.29	1.48	4.34
Unidentified	5.43	1.78	3.25	5.61	9.70	6.78	7.43	3.00	0.65	5.13
24:0 + 24:1	41.13	36.60	39.92	37.10	44.60	41.41	39.30	35.58	41.94	40.64
24:1/24:0	0.70	0.65	0.50	0.49	0.43	0.42	0.39	0.37	0.29	0.29

[a]Values are means of n = 3, expressed as mol%. ND, <0.005 mol%. See Table 1 for other abbreviations.

supplemented diets resulted in significant variation (Tukey's test, $P < 0.05$) in 18:1, 20:0, 22:0, 24:0, and 24:1 in SM. Stepwise multiple linear regression analysis between the fatty acid ethyl esters in the diet and the fatty acids in SM, shown in Table 5, gave the following equation: $y = 1.42\ (24:1) - 0.23\ (24:0) + 0.50\ (22:1) + 0.17\ (20:1) + 0.61$, $r^2 = 0.98$, $P < 0.0001$. The plot of predicted vs. observed effects is shown in Figure 2. As in experiment 1, 24:1 is the largest positive factor and 24:0 is the largest negative factor in the regression formula.

DISCUSSION

The results of these experiments demonstrate clearly that dietary fat type can alter rapidly the 24:1/24:0 ratio in SM in rat liver over a tenfold range. Multiple regression analysis of fatty acid composition of the diet with rat liver SM fatty acid composition demonstrates that some very long chain fatty acids, particularly 22:1, 24:0 and 24:1, were potent factors in influencing the 24:1/24:0 ratio in liver SM. Our data indicate

TABLE 3
Summary of Stepwise Multiple Regression of Diets Supplemented with Different Dietary Oils

	Fatty acids[a]							
	22:1	24:0	24:1	18:1	22:0	20:1		
	Regression coefficients						Intercept	r^2
Step 1	0.15[b]						0.77	0.5754
Step 2	0.26	–1.65					0.80	0.8037
Step 3	0.23	–1.72	1.54				0.70	0.9000
Step 4	0.21	–1.49	1.88	0.012			0.26	0.9500
Final	0.25	–1.45	2.13	0.0083	0.33	–0.074	0.33	0.9741

[a]Fatty acids are expressed as mol%.
[b]$P < 0.0001$ for all regression coefficients, intercept values, and each step. No other regression coefficients were significant at the $P < 0.1000$ level.

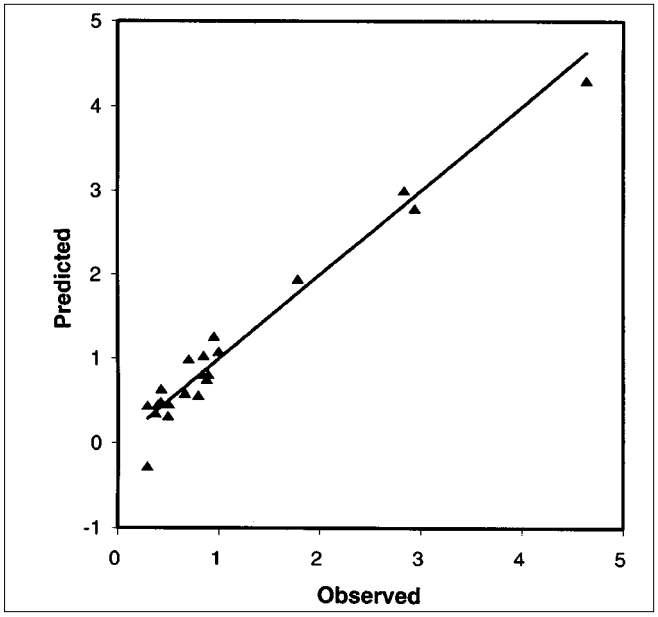

FIG. 1. Predicted versus observed values for the formula $y = 1.88(24:1) - 1.49(24:0) + 0.21(22:1) + 0.012(18:1) + 0.26$, ($r^2 = .95$, $P < 0.0001$); for the relationship between the 24:1/24:0 sphingomyelin ratio and the mol% fatty acid composition of the dietary oils.

that 24:1 and 24:0, when present in trace [<0.1% (w/w)] amounts in the diet, significantly influence liver SM fatty acid composition.

The mathematical model resulting from our experiments helps to understand dietary fat-induced changes in the 24:1 and/or 24:0 in tissue SM reported by other investigators. For example, dietary intake of high erucic acid rapeseed oil increased the 24:1 and decreased the 24:0 content of SM in lung (11) and heart (7) of rats and in heart of swine (5); rats fed a low erucic acid rapeseed oil also had increases in 24:1 and decreases in 24:0 in heart SM relative to rats fed peanut oil (12). Mice fed diets containing peanut oil had higher 24:0 in liver SM than mice fed diets containing olive oil (2); mice fed diets containing borage oil had higher 24:1 and lower 24:0 in kidney SM than mice fed diets containing corn oil (13). Humans given supplements of borage oil (3) or cod liver oil (14) had significant increases of 24:1 in platelet SM. Patients with adrenoleukodystrophy given supplements of Lorenzo's oil had a dramatic increase in 24:1 and decrease in 24:0 in plasma SM (6). Our mathematical model, based on a rat liver bioassay, appears to be useful in predicting effects of dietary fatty acid intake on 24:1/24:0 ratio of SM in multiple species and in multiple tissues.

Our data indicate that dietary very long chain fatty acids are not the only dietary fatty acids, or fat components, to alter SM fatty acid composition. Dietary 18:1 was a significant positive factor in predicting the 24:1/24:0 ratio in liver SM but did not correlate with the 18:1 content of SM. This result is similar to that found by Neelands and Clandinin (15) in rat liver plasma membranes. Similarly, 18:2n-6 was a significant positive factor ($r^2 = 0.35$, $P < 0.0001$) in predicting the 24:2n-6 content of SM but did not correlate with the 18:2n-6 content of SM; PUFA-rich diets have been reported to increase the 24:2n-6 content of SM in canine erythrocytes and platelets and in swine erythrocytes (4).

The present experiments illustrate the need for more research in two areas regarding the effects of dietary fat on SM metabolism and function: the mechanism(s) by which dietary fatty acid intake alters SM fatty acid composition and the physiological effects of diet-induced changes in SM fatty acid composition. Moreover, the data suggest that further study on the nutritional and/or pharmacological value of 24:1 and 24:0 in humans and animals is warranted.

TABLE 4
Sphingomyelin Fatty Acid Composition of Livers from Rats Fed Diets Supplemented with Ethyl Esters of Fatty Acids[a]

	Diets[b]					
Fatty acid	24:1	22:1	20:1	20:0	22:0	24:0
16:0	20.48	22.58	31.47	26.27	24.91	25.88
18:0	5.98	6.28	6.67	9.67	4.56	8.95
18:1	1.78	1.77	1.42	0.73	2.96	4.40
18:2	1.06	2.03	2.30	2.70	1.37	2.21
20:0	1.54	1.60	1.67	3.17	1.52	1.36
22:0	5.05	7.46	7.46	12.77	17.61	4.64
22:1	1.21	0.75	1.95	0.62	0.39	1.17
23:0	4.18	5.55	5.71	5.73	4.48	3.39
iso 24:0	2.22	3.48	5.97	2.41	2.02	2.50
24:0	15.46	20.26	18.76	21.63	25.80	31.76
24:1	40.85	28.25	16.63	14.36	14.29	10.07
Unidentified	ND	1.05	ND	7.98	1.07	9.78
24:0 + 24:1	56.30	48.51	35.40	35.92	40.17	48.83
24:1/24:0	2.64	1.40	0.92	0.67	0.56	0.32

[a]Values are the means of $n = 3$ expressed as mol%.
[b]Diets contained 5.0 wt% olive oil supplemented with 1.0 g of the ethyl esters indicated per kg of diet. See Table 1 for abbreviation.

TABLE 5
Summary of Stepwise Multiple Regression of Ethyl Ester Supplemented Diets

	Fatty acids[a]					
	24:1	22:1	24:0	20:1		
	Regression coefficients				Intercept	r^2
Step 1	1.33[b]				0.76	0.7981
Step 2	1.42	0.50			0.64	0.9267
Step 3	1.37	0.45	–0.28		0.72	0.9598
Final	1.42	0.50	–0.23[c]	0.17[c]	0.61	0.9761

[a]Fatty acids are expressed as mol%.
[b]$P < 0.0001$ for all regression coefficients, intercept values, and each step.
[c]$P < 0.005$. No other regression coefficients were significant at the $P < 0.1000$ level.

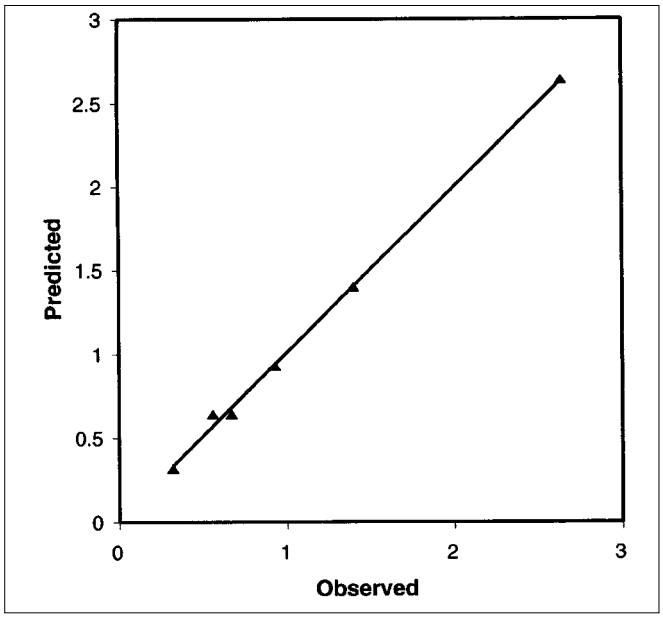

FIG. 2. Predicted versus observed values for the formula $y = 1.42(24:1) - 0.23(24:0) + 0.50(22:1) + 0.17(20:1) + 0.61$, ($r^2 = .98$, $P < 0.0001$); for the relationship between the 24:1/24:0 sphingomyelin ratio and the mol% fatty acid composition of the diets supplemented with ethyl esters.

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Androgen Regulation of Lung Lipids in the Male Rat

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ABSTRACT: The aim of this study was to determine whether or not testosterone regulates the lipid concentration in rat lung tissue. Rats were either sham-operated controls, castrated, or castrated and injected with testosterone. Twenty-one days after castration, we observed in relation to the control: (i) Total lipids, phospholipids, and total cholesterol increased, while triglycerides decreased in whole lung. (ii) Phospholipid concentration increased in microsomes, lamellar bodies, and alveolar macrophages, but it decreased in extracellular surfactant. (iii) On a percentage basis, the concentration of phosphatidylcholine increased in microsomes, lamellar bodies, and alveolar macrophages, and it decreased in extracellular surfactant. (iv) Protein concentration decreased in extracellular surfactant and increased in microsomes, lamellar bodies, and alveolar macrophages. (v) The incorporation of [^{14}C]glycerol into phospholipids of lung slices increased. (vi) The activity of CTP:phosphocholine cytidylyltransferase bound to the microsomal fraction increased without any change in the activity of the soluble form of the enzyme in the lung. The results obtained when testosterone was administered to castrated rats were similar to those obtained in the control in all cases. These results suggest that the lipid concentration in the lung is regulated at least partly directly or indirectly by androgens.
Lipids 32, 57–62 (1997).

Sex-related differences in fetal lung development are now widely recognized. In various species, it is clear that lung maturation occurs sooner in females than in males (1). Male infants are at a greater risk of developing respiratory distress syndrome than females (2). It is known that the administration of dihydrotestosterone to pregnant rabbits during the course of fetal sexual differentiation eliminates the difference by inhibiting surfactant production in the female fetus (3). There are several reports of attempts to elucidate the mechanism responsible for sex differences in pulmonary surfactant production in fetal lung (4). However, there is no report on the effect of testosterone on the lipid composition of lung tissue in adult male rats (5). In this study we measured the amount of different lipids in adult male rat lung after castration in order to determine whether or not they are under testosterone regulation. We report evidence that testosterone regulates the amount of lipids in the adult male rat.

MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade. Standards of cholesterol, triglycerides, phospholipids, and protein were purchased from Sigma Chemical Co. (St. Louis, MO). Testosterone enantate was obtained from Testosteron Depot Schering A.G. (Berlin, Germany). Serum total lipids, cholesterol, and triglycerides were determined using assay kits from Wiener Laboratory (Rosario, Argentina). [U-^{14}C] Glycerol, 3700 M Bq/mmol, 10.0 mCi/mmol and [Me-^{14}C] phosphorylcholine, 3.7 M Bq/mL, 50 mCi/mmol were obtained from New England Nuclear Corp. (Boston, MA).

Animals and feeding procedure. Wistar adult male rats, 4 months old, 200 g weight, were separated into three groups. The first group was sham-operated and used as control (Co); the second was subjected to castration by simple orchidectomy (Ca); and the third received by intramuscular (i.m.) injection testosterone (100 μg/kg body weight) 15 d after castration (Ca + T). Twenty-one days after castration, all rats were anesthetized by an i.m. injection of urethane (250 mg/100 g body weight in 1 mL of saline solution). The lungs were quickly removed, lavaged with ice-cold 0.9% saline solution, and weighed.

Isolation of extracellular pulmonary surfactant and macrophages. In another experiment, the trachea of anesthetized rats was cannulated, and the lungs were filled with 2.5 mL ice-cold 0.9% saline solution. Lavages containing the extracellular surfactant were then collected. This procedure was repeated nine times with fresh 0.9% saline. The combined lavages were centrifuged at 4°C and 580 × *g* for 10 min to sediment macrophages. The resulting supernatant was centrifuged at 100,000 × *g* for 1 h in a Beckman ultracentrifuge (LS 65 B, 65 Ty rotor; Fullerton, CA) to obtain a surfactant pellet (6).

Isolation of lamellar bodies and microsomes. After obtaining the extracellular pulmonary surfactant fraction, the lavaged lungs were used to isolate lamellar bodies and microsomes (6).

Lipid analyses. Lipids were extracted from total lung tissue, microsomes, lamellar bodies, extracellular surfactant, and

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Abbreviations: Ca, castrated rat; Co, control rat; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI + PS, phosphatidylinositol + phosphatidylserine; Sph, sphingomyelin; TLC, thin-layer chromatography.

macrophages (7). Total lipids were determined by dry weight. The lipids were resuspended in chloroform/methanol 2:1, vol/vol. Aliquots were taken for determining phospholipids and measuring phosphorus (8) and total cholesterol (9). Other parts of the extract were used for the separation of the different lipids on thin-layer chromatography (TLC) plates coated with silica gel G (Merck, Darmstadt, Germany) using hexane/diethyl ether/acetic acid (80:20:1, by vol) as solvent. The lipids were detected by exposing the plates to iodine vapors. They were scraped off and used directly for determinations of phospholipids (8), triglycerides (10), free and esterified cholesterol (11).

Analysis of phospholipid composition. Phospholipids were separated into component species by TLC using silica gel H plates and chloroform/methanol/water (65:25:4, by vol) as solvent (12). The individual phospholipids were identified and recovered as above and quantified (8). The position of the phospholipids was determined using the respective standards. The results were expressed as the percentage of total phospholipid phosphorus.

Incorporation of [U-^{14}C] glycerol into phospholipids. The lung slices (250–350 mg) were immediately placed in individual flasks containing 4 mL Krebs-Ringer bicarbonate solution and 0.6 mL of 0.1 mM glucose (pH 7.4). The medium was aerated with a 95:5 mixture of O_2/CO_2. Slices were preincubated for 5 min at 37°C in a Dubnoff metabolic shaking incubator. [^{14}C] glycerol was added (1 mCi/incubation flask) to start the reaction. After 60 min of incubation, the reaction was stopped by placing the flask in an ice bath. The lipids were extracted (7).

Duplicate samples (10 mL each) were used for the separation of the different labeled phospholipids by TLC. The different phospholipid spots were detected using iodine vapors. In each case only one sample was used for determining radioactivity in a Beckman LS 100 C liquid scintillation counter, and the other sample was used for determining phosphorus (8). Quantitative recovery of each phospholipid component was about 98% .

CTP:phosphocholine cytidylyltransferase assay. Lungs of castrated and noncastrated rats were used for the separation of cytosol and microsomes for enzymatic determination (13). Assay mixtures contained 20 mM Tris/succinate pH 7.8, 6 mM $MgCl_2$, 8 mM CTP, and 4 mM [Me-^{14}C] phosphorylcholine (sp. radioactivity 0.15 μCi/μmol). The reaction was started by the addition of enzyme (100 μg protein), and the assay mixture was incubated for 30 min at 37°C in a reaction volume of 200 μL. The reaction was stopped by boiling for 2 min, and the protein was removed by centrifugation (1000 × *g* for 10 min). A 50-μL sample was taken from the supernatant, mixed with 2 nmol of unlabeled phosphorylcholine as carrier, and subjected to TLC on precoated silica gel G 60 (Merck) with methanol: NaCl 0.15 M: conc. NH_4OH (50:50:5, by vol) as solvent for the isolation of the reaction product. The CDP-choline spots were detected with iodine vapor, scraped off the plates into scintillation vials, and assayed for radioactivity. Under these conditions, the reaction rate was linear over time and with protein concentration.

Electron microscopy. The pellets of isolated lamellar body and microsomes were fixed with 2.5% glutaraldehyde and analyzed (14).

Enzyme markers. To determine if lamellar body was contaminated with microsomal mitochondrial or cell membranes, the activities of NADPH-cytochrome c reductase (16), succinate dehydrogenase (17) and 5′ nucleotidase (18), respectively, were measured.

Protein assay. Protein content was determined by the Layne method (14) using fraction V bovine serum albumin as standard.

Statistical analysis. Results are expressed as means ± standard deviations. Statistical differences were tested by analysis of variance test and Tukey test.

RESULTS

Effect of castration on body weight and lung weight. After 21 d of castration, the gain of weight was lower in Ca rats compared to controls. The lung weight of Ca rat was higher than that of Co. When testosterone was administrated to Ca, the lung weight was similar to that of the control (Table 1).

Total lipids, cholesterol, and triglycerides in serum and whole lung. Total lipids and triglycerides decreased while cholesterol increased in the serum of Ca compared to that in Co. Total lipids and total free cholesterol increased, esterified cholesterol did not change, and triglycerides decreased in whole lung of Ca compared to Co (Table 1).

TABLE 1
Body and Lung Weights, and Lipids from Serum and Lung of Male Rat[a]

	Control	Castrated	Castrated testosterone
Body weight (g)			
Initial	288 ± 26	287.5 ± 27	280 ± 32
Final	325 ± 30	315 ± 31	320 ± 27
Gain	43 ± 13^b	23.83 ± 9^c	33 ± 7^b
Lung weight (g)	1.66 ± 0.3^d	1.91 ± 0.2^e	1.63 ± 0.2^d
Serum (g/L)			
Total lipids	4.70 ± 0.80^d	2.07 ± 0.70^e	n.d.
Cholesterol	0.94 ± 0.13^d	1.60 ± 0.30^e	1.25 ± 0.30^d
Triglycerides	1.00 ± 0.30^d	0.40 ± 0.10^e	n.d.
Whole lung (mg/g lung)			
Total lipids	65 ± 4.7^b	81.00 ± 3.1^n	65 ± 7.6^b
Cholesterol			
Total	6.50 ± 1.6^d	8.50 ± 1.6^e	n.d.
Free	4.30 ± 0.8^d	6.50 ± 1.2^e	n.d.
Esters	0.70 ± 0.16	0.94 ± 0.16	n.d.
Triglycerides	0.305 ± 0.08^d	0.191 ± 0.02^e	0.316 ± 0.01^d
Macrophages (mg/g lung)			
Total cholesterol	0.055 ± 0.00^b	0.093 ± 0.00^n	0.056 ± 0.00^b

[a]Results given as mean ± SD; $n = 12$ for each case. Across a row, values with different superscript letter indicate significant difference, by analysis of variance and Tukey test. $^{b,c}P < 0.05$; $^{d,e}P < 0.01$; $^{b,n}P < 0.001$; n.d., not determined.

Phospholipid concentration in intracellular and extracellular compartments of lung. The phospholipid concentration increased in whole lung, microsomes, and lamellar bodies, while in extracellular surfactant it decreased in Ca compared to Co (Table 2).

On a percentage basis, the phospholipid composition in whole lung and other particles studied was modified after castration compared to that in control animals. In whole lung, phosphatidylcholine (PC), phosphatidylserine (PS) + phosphatidylinositol (PI), lysophosphatidylcholine (LPC) and phosphatidylglycerol (PG) increased; sphingomyelin (Sph) and phosphatidylethanolamine (PE) decreased. In microsomes PC and PE increased, PI + PS and LPC decreased, but Sph and PG were not changed (Table 3). In lamellar bodies, PC and PS + PI increased; LPC, Sph, PE, and PG decreased. In extracellular surfactant, after castration the amount of PC and PE decreased and LPC, Sph, PI + PS increased compared to controls. In alveolar macrophages LPC, Sph, PC, and PG increased while PE decreased and PS + PI did not change (Table 3).

Incoporation of [U- ^{14}C] glycerol into phospholipids of lung slices. The specific activity of PC, PI + PS, PE, LPC, and PG increased in Ca compared to Co, while the specific activity of Sph was not modified by castration in relation to that in the control (Table 4).

Activity of CTP:phosphocholine cytidylyltransferase. The enzyme activity binding to endoplasmic reticulum membranes increased, but it did not change in the cytosol of Ca (Table 2).

Protein concentration. Protein concentration did not change in whole lung. It increased in microsomes, lamellar bodies, and alveolar macrophages and decreased in extracellular surfactant (Table 2).

Electron microscopy and enzyme markers. Analysis by electronic microscopy showed that 95% of the counted particles were lamellar bodies and microsomes. The activities of marker enzymes showed that the isolated fractions were 98% pure.

DISCUSSION

While the role of androgens in the maturation of the fetal lung has received much attention, the role of these hormones in the adult rat lung has not been studied. Androgen receptors are present in the lung, and the function of these receptors in lung maturation has been demostrated in mice with the testicular feminization syndrome, in which androgen receptors are absent (19). Our results obtained in the different fractions of lungs show that castration could cause changes in the synthesis, secretion, and clearance of phospholipids in surfactant. Lipids and protein of surfactant are synthesized by alveolar type II cells, and they are stored prior to secretion in intracellular organelles called lamellar bodies (20).

In our experiment, the low gain of body weight observed in Ca compared to Co confirms previous results (21). The higher lung weight of Ca compared to Co could be a consequence of accumulation of substances as total lipids, as it has been in our experiments.

TABLE 2
Phospholipids, Proteins, and Activity of CTP-Phosphocholine Cytidylyltransferase of Lung[a]

	Control	Castrated	Castrated + testosterone
Phospholipids (mg/g lung)			
Whole lung	23.25 ± 3.10^{b}	35.68 ± 4.6^{n}	29.68 ± 3.10^{b}
Microsomes	3.72 ± 1.55^{c}	7.75 ± 2.48^{d}	n.d.
Lamellar bodies	13.02 ± 2.17^{b}	23.87 ± 3.10^{n}	19.22 ± 0.93^{b}
Surfactant	8.37 ± 2.17^{c}	4.96 ± 1.24^{d}	8.06 ± 1.24^{c}
Macrophages	1.86 ± 0.96^{c}	3.41 ± 0.34^{d}	1.24 ± 0.31^{c}
Proteins (mg/g lung)			
Whole lung	67.01 ± 5.79	60.78 ± 8.02	58.15 ± 1.84
Microsomes	0.20 ± 0.03^{b}	0.88 ± 0.21^{n}	0.21 ± 0.03^{b}
Lamellar bodies	5.02 ± 0.80^{b}	6.1 ± 0.64^{c}	5.24 ± 0.7^{b}
Surfactant	1.77 ± 0.27^{b}	1.10 ± 0.2^{n}	1.62 ± 0.21^{b}
Macrophages	1.27 ± 0.05^{b}	1.76 ± 0.12^{n}	1.23 ± 0.05^{b}
CTP-phosphocholine cytidylyltransferase activity (dpm/min/mg prot.)			
Microsomal fraction	12959 ± 200^{b}	2678 ± 451^{n}	n.d.
Soluble fraction	320 ± 105	390 ± 120	n.d.

[a]Results given as mean ± SD; $n = 12$ for each case. Across a row, values with different superscript letter indicate significant difference, by analysis of variance and Tukey test. $^{b,c}P < 0.05$; $^{c,d}P < 0.01$; $^{b,n}P < 0.001$; n.d., not determined.

TABLE 3
Phospholipid Composition of Lung and Macrophages of Male Rat[a]

	LPC	Sph	PC	PS + PI	PE	PG
Whole lung						
Co	1.9 ± 0.1[b]	13.0 ± 2.3[b]	52.1 ± 2.3[b]	6.7 ± 0.4[b]	15.1 ± 2.8[b]	4.7 ± 0.1[b]
Ca	2.4 ± 0.1[e]	7.2 ± 1.3[e]	68.7 ± 1.6[e]	12.1 ± 2.7[e]	6.7 ± 1.5[e]	5.9 ± 0.0[e]
Ca + T	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Microsomes						
Co	1.5 ± 0.1[b]	10.7 ± 1.9	68.2 ± 1.5[b]	13.0 ± 0.3[b]	1.2 ± 0.0[b]	4.4 ± 0.2
Ca	0.7 ± 0.0[e]	9.4 ± 0.3	80.0 ± 0.4[e]	4.5 ± 0.2[e]	4.2 ± 0.3[e]	4.0 ± 0.1
Ca + T	1.5 ± 0.0[b]	12.0 ± 1.7	69.3 ± 2.0[b]	12.8 ± 0.7[b]	1.7 ± 0.5[b]	4.8 ± 0.1
Lamellar body						
Co	0.7 ± 0.0[b]	5.3 ± 0.3[b]	68.1 ± 1.2[b]	4.5 ± 0.5[b]	11.7 ± 0.2[b]	5.4 ± 0.1[b]
Ca	0.5 ± 0.0[e]	2.5 ± 0.7[e]	77.5 ± 2.5[e]	8.8 ± 1.4[e]	7.3 ± 0.2[e]	4.3 ± 0.3[e]
Ca + T	0.7 ± 0.0[b]	7.3 ± 1.2[b]	70.1 ± 2.2[b]	4.8 ± 0.4[b]	10.9 ± 1.1[b]	5.2 ± 0.2[b]
Extracelullar surfactant						
Co	0.4 ± 0.0[b]	0.9 ± 0.0[b]	82.6 ± 8.4[c]	2.5 ± 0.3[b]	6.5 ± 0.6[c]	8.8 ± 0.5[b]
Ca	0.7 ± 0.0[e]	1.7 ± 0.1[e]	68.5 ± 1.2[d]	10.7 ± 0.7[e]	4.0 ± 0.3[d]	14.9 ± 0.8[e]
Ca + T	0.4 ± 0.0[b]	0.8 ± 0.0[b]	80.6 ± 4.2[c]	3.3 ± 0.2[b]	6.6 ± 0.1[c]	9.2 ± 1.3[b]
Alveolar macrophages						
Co	0.7 ± 0.0[c]	3.1 ± 0.2[b]	68.3 ± 2.8[c]	9.7 ± 0.7	10.7 ± 2.0[b]	6.4 ± 0.8
Ca	0.8 ± 0.0[d]	6.4 ± 1.4[e]	72.9 ± 3.4[n]	8.1 ± 1.4	2.1 ± 0.4[e]	9.7 ± 1.2
Ca + T	0.7 ± 0.0[c]	3.8 ± 0.8[b]	66.6 ± 3.7[c]	10.2 ± 1.2	10.6 ± 1.7[b]	7.1 ± 2.2

[a]Results given as mean ± SD; $n = 6$ for each group. Co, control; Ca, castrated; Ca + T, castrated + testosterone; LPC, lysophosphatidylcholine; Sph, sphingomyelin; PC, phosphatidylcholine; PS + PI, phosphatidylserine + phosphatidylinositol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol. Across a row values with different superscript letter indicate significant difference by analysis of variance and Tukey test. [b,e]$P < 0.001$, [c,d]$P < 0.01$, [c,n]$P < 0.05$; n.d., not determined.

TABLE 4
Incorporation of ^{14}C-Glycerol in Phospholipids of Lung Slice After Castration[a]

	Specific activity (dpm/mmol P)	
Phospholipids	Control	Castrated
Lysophosphatidylcholine	12288 ± 1540[d]	15552 ± 1800[n]
Sphingomyelin	450 ± 95	380 ± 210
Phosphatidylcholine	234183 ± 1286[c]	267513 ± 1630[b]
Phosphatidylserine + phosphatidylinositol	64267 ± 2320[c]	82973 ± 5300[b]
Phosphatidylethanolamine	24069 ± 1180[c]	41744 ± 1030[b]
Phosphatidylglycerol	42727 ± 4700[c]	50239 ± 2000[b]

[a]Results given as mean ± SD; $n = 11$ for each group. Across a row values with different superscript letter indicate significant difference by analysis of variance and Tukey test. [c,b]$P < 0.001$, [d,n]$P < 0.01$.

It is known that castration increases the cholesterol concentration in serum (22). Cholesterol is the major neutral lipid component of extracellular surfactant, and it is accepted that most cholesterol in the lung is taken up from circulating low density (LDL) or high density lipoprotein (HDL) (23). The increment of cholesterol in serum could be a cause of the increased cholesterol concentration observed in whole lung and macrophages in Ca compared to Co. Studies utilizing isolated perfused rat lung have demonstrated the uptake of very low density lipoproteins from the perfusion medium (24).

The decreased triglyceride concentration observed in the whole lung of Ca could be a consequence of more utilization of this as precursor for synthesizing phospholipids or it could be a consequence of decreased triglyceride concentration observed in serum of Ca. In our laboratory we have found that the synthesis of triglycerides decreased in liver after castration (25).

The protein concentration was not modified in whole lung by castration, probably because the lung has more than 40 different types of cells, and androgen could have different regulatory effects on each one. However, we observed an increment in the protein concentration in microsomes, lamellar bodies, and macrophages. These results are probably associated with changes in the concentration of specific proteins. The low protein concentration of the extracellular surfactant could be a consequence of decreased secretion of this from lamellar bodies or decreased synthesis of specific protein of extracellular surfactant. We could infer that the phospholipid increment in microsomes and lamellar bodies in lungs of rats could be a consequence of a greater synthe-

sis in microsomes with transfer to lamellar bodies. There was more incorporation of [^{14}C] glycerol into phospholipids as well as an increment in the activity of CTP:phosphocholine cytidylyltransferase, the regulatory enzyme of PC synthesis. The activity of this enzyme is regulated by various effector molecules (26,27). The lower content of phospholipid in extracellular surfactant may be a consequence of greater uptake by macrophages or increased recycling by neumocytes type II for synthesis of new surfactant (28,29). In our experiment we observed an increased phospholipid concentration in alveolar macrophages of Ca compared to Co. This increment may be due to two causes: (i) An increment in the removal rate of surfactant. Evidence has been obtained which suggests that alveolar macrophages phagocytize surfactant material and that some of the surfactant lipids may be degraded (30). (ii) An increment in the amount of cholesterol that increases the synthesis of PC through stimulation of CTP:phosphocholine cytidylyltransferase (31). In our experiment we observed simultaneously an increment of cholesterol and phospholipids in macrophages from Ca compared to Co.

At this time we do not know if the increased activity of CTP:phosphocholine cytidylyltransferase is due to a pre- or posttranslational regulation. It may be that the increment in cholesterol concentration is an indirect cause of the increased activity of this enzyme observed in lung of Ca. We believe that androgen may be an important regulator of lipid synthesis in lung directly or indirectly.

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Visual Acuity and Blood Lipids in Term Infants Fed Human Milk or Formulae

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ABSTRACT: This multicenter, parallel group study determined plasma phospholipid and red blood cell (RBC) phosphatidylcholine and phosphatidylethanolamine fatty acids, plasma cholesterol, apo A-1 and B, growth and visual acuity (using the acuity card procedure) in term infants fed from birth to 90 d of age with formula containing palm-olein, high oleic sunflower, coconut and soy oil (22.2% 16:0, 36.2% 18:1, 18% 18:2n-6, 1.9% 18:3n-3) (n = 59) or coconut and soy oil (10.3% 16:0 18:6% 18:1, 34.2% 18:2n-6, 4.7% 18:3n-3) (n = 57) or breast-fed (n = 56) with no formula supplementation. Different centers in North America were included to overcome potential bias due to differences in n-6 or n-3 fatty acids at birth or in breast-fed infants that might occur in a single-site study. Plasma and RBC phospholipid docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6), cholesterol and apo B were significantly lower in the formula- than breast-fed infants. There were no differences in looking acuity or growth among the breast-fed and formula-fed infants. No significant relations were found between DHA and looking acuity, or AA and growth within or among any of the infant groups. This study provides no evidence to suggest the formula provided inadequate n-6 or n-3 fatty acids for growth and looking acuity for the first 3 mon after birth.

Lipids 32, 63–72 (1997).

The physiological importance of docosahexaenoic acid (DHA, 22:6n-3) in normal visual and brain function is now recognized (1–4). Several studies have shown that the brain and eye contain all the enzymes necessary for synthesis of DHA from the dietary essential fatty acid α-linolenic acid (LNA, 18:3n-3) (5–7). Other studies, however, have shown DHA from plasma is taken up and incorporated into central nervous system (CNS) lipids (2,8). Plasma DHA can be derived from DHA in the diet, or from DHA synthesized from LNA in the liver and secreted in lipoproteins. Arachidonic acid (AA, 20:4n-6) is also present in high concentrations in CNS phospholipids and is important in many biochemical pathways including eicosanoid metabolism (2). AA can be synthesized from the dietary essential fatty acid linoleic acid (LA, 18:2n-6), but as for DHA, the pathway by which developing brain normally acquires AA is not clear.

Synthesis of DHA requires adequate activity of the fatty acid desaturase enzymes, an adequate dietary intake of LNA, and an appropriate balance of LA to LNA (9). Recent studies have shown conversion of LNA to DHA, and LA to AA in term and preterm infants (10,11). It is not clear, however, if the rates of DHA and AA synthesis are sufficient to meet the tissue needs of young infants.

Human milk contains small but variable amounts of DHA, usually ranging from about 0.2–0.4% fatty acids, and about 0.5% fatty acids as AA (11). Infant formulae containing vegetable and oleo oils contain LA and LNA but, in contrast to human milk, infant formulae in North America have no preformed AA or DHA. Term and preterm infants fed formula containing no AA or DHA have lower blood lipid levels of AA and DHA than infants fed human milk (12–15). The physiological significance of the lower blood lipid DHA and AA in formula-fed than breast-fed term gestation infants is controversial. Some studies have found no differences in tests of visual acuity or in electrophysiological tests of visual function between breast-fed and formula-fed term gestation infants (12,16,17). Others, however, have found evidence of lower visual function in term infants fed formula without DHA than in breast-fed infants (13,18–20), with no significant difference in visual function between infants fed formula with DHA and those who were breast-fed (20). The reason for the discrepancy in the results of different studies is not yet clear.

Studies with very premature infants have suggested an inverse relationship between blood lipid AA and growth (21). In other recent work, a potential negative effect of increasing LNA in formula on growth was suggested (22). Few previous studies have given a direct comparison of growth between infants fed formula with higher compared to lower levels of LNA. Differences in growth, however, have not been found in other recent studies with infants fed formula or breast-fed from birth to 3–4 mon of age (12,14,23).

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Abbreviations: AA, arachidonic acid; CNS, central nervous system; DHA, docosahexaenoic acid; LA, linoleic acid; LNA, linolenic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RBC, red blood cell; VEP, visual evoked potential.

The objective of this study was to determine the effect of infant formula containing differing amounts of LA and LNA with no AA or DHA on the blood lipid fatty acids, preferential looking acuity, and growth of healthy term gestation infants, with reference to normal breast-fed infants. Because the formulae studied also differed in levels of saturated, monounsaturated and polyunsaturated fatty acids, the effects on plasma cholesterol and apolipoprotein (apo) A-I and apoB were also measured. The study was undertaken at several centers in the United States and Canada selected to represent a typical North American breast-feeding population. The study included measures of growth through 90 d of age, analysis of plasma and erythrocyte phospholipid fatty acids at 14 and 90 d of age, and looking acuity, utilizing the Acuity Card Procedure at 90 d of age. The study duration was 90 d because solid foods are typically introduced between 3 and 4 mon after birth, and birth to 3 mon is the time of most rapid development of preferential looking acuity (24,25).

MATERIALS AND METHODS

Infants and diets. This was a multicenter, parallel-group study conducted simultaneously at seven different centers in the United States and Canada, with all the centers following a common study protocol. Infants were eligible to participate if they were term gestation, 37–41 wk gestation with a birth weight >2500 g to <4500 g, were less than 14 d of age, and if the mother had chosen to exclusively breast-feed or feed formula for at least 3 mon. Breast-fed infants who received formula later than 6 d after birth were not eligible. Infants with congenital problems or disease considered likely to interfere with normal feeding or nutrient metabolism, with feeding intolerance, poor milk or formula intake, or with abnormal eye exam, as judged by the infant's physician were also ineligible. Formula-fed infants were randomly assigned to receive one of two cow milk-protein based formulae (Mead Johnson Nutritionals, Evansville, IN) which differed only in the fat blend and fatty acid composition (Table 1). The computer-generated randomization schedules used to assign formula-fed infants to one of the two formulae were prepared centrally and distributed to each participating center. Formula 1 (Enfamil; Mead Johnson Nutritionals) had (percentage total fatty acids) 18.0% linoleic acid (LA, 18:2n-6), 1.9% linolenic acid (LNA, 18:3n-3) with an LA/LNA ratio of 9.5:1. Formula 2 had 34.2% 18:2n-6 and 4.7% 18:3n-3 with an LA/LNA ratio of 7.3:1 (Table 1). Neither formula had any detectable AA, DHA, or other carbon chain (C) 20 or 22 n-6 or n-3 fatty acids.

Breast-fed infants were breast-fed from birth, and from 6 d after birth could receive no infant formula for the first 30 d of the study, then no more than 6 ounces of formula per day. The group of breast-fed infants was subsequently stratified to those who received no supplemental formula and those who did. The parents were instructed not to feed solid foods and to record any incidence of feeding with anything other than the assigned formula or breast milk. Infants who received more than 10% dietary energy from sources other than the assigned formula or breast milk for 5 d or more were withdrawn from the study. The infants were monitored for any adverse reactions, concomitant medications, and illnesses or symptoms of concern. Infants who had formula intolerance, who were not brought for blood sampling, or with illness or other

TABLE 1
Major Fatty Acid Components (weight percentage total fatty acids) of Human Milk and Formula[a]

Fatty acid	Human milk 14 d $n = 55$	Human milk 90 d $n = 56$	Formula 1	Formula 2
12:0	5.6 ± 2.3	5.1 ± 1.7	9.3	16.8
14:0	5.8 ± 2.0	5.8 ± 1.6	5.2	7.8
16:0	21.4 ± 2.0	21.2 ± 2.4	22.2	10.3
16:1	3.0 ± 0.9	2.9 ± 0.8	0.2	0.1
18:0	7.7 ± 1.5	8.1 ± 1.7	5.2	4.6
18:1	36.4 ± 3.9	37.3 ± 4.6	36.2	18.6
18:2n-6	14.4 ± 3.9	14.6 ± 4.2	18.0	34.2
18:3n-3	1.0 ± 0.4	1.2 ± 0.6[b]	1.9	4.7
20:2n-6	0.5 ± 0.1	0.3 ± 0.1[b]	0.1	0.1
20:3n-6	0.5 ± 0.1	0.3 ± 0.1[b]	n.d.	n.d.
20:4n-6	0.6 ± 0.1	0.5 ± 0.1[b]	n.d.	n.d.
20:5n-3	<0.1	<0.1	n.d.	n.d.
22:4n-6	0.1 ± 0.0	0.1 ± 0.0[b]	n.d.	n.d.
22:5n-3	0.1 ± 0.0	0.1 ± 0.0	n.d.	n.d.
22:5n-6	0.1 ± 0.0	<0.1	n.d.	n.d.
22:6n-3	0.3 ± 0.1	0.2 ± 0.1[b]	n.d.	n.d.

[a]Values for human milk are means ± SD, and for the formulae are the average of three separate analyses for each.
[b]Value for human milk on day 90 significantly different from value for the same fatty acid on day 14, $P < 0.01$.
n.d., Not detected. <0.1 indicates value of >0 to ≤0.05.

circumstances which resulted in dietary intakes <50% of normal for 5 d or more were withdrawn. The study protocol and procedures were reviewed and approved by the Institutional Review Board at each site at which infants were enrolled. Informed, written consent was obtained from a parent for all infants prior to participation.

Growth and blood collection. Body weight, length, and head circumference were recorded for all infants at birth, enrollment, 1, 2 and 3 mon of age. Breast-feeding mothers provided a midfeed sample of breast milk at 14 d and 3 mon postpartum. The milk samples were immediately frozen then transferred to storage at −70°C. Blood samples were collected by venipuncture with EDTA as the anticoagulant from each infant at 14 d and at 90 ± 4 d (3 mon) of age and within 2 h of feeding. The tubes containing the blood samples were immediately placed on ice, then the plasma and erythrocytes separated by centrifugation. The erythrocyte pellet was washed and repelleted, and the plasma and erythrocyte pellets were stored at −70°C prior to shipment to Vancouver for analysis of fatty acids (12,14).

Visual acuity. Binocular preferential looking (visual) acuity was tested at 90 ± 4 d of age using the acuity card procedure (26) with Acuity Cards from Vistech, Inc. (Dayton, OH) essentially as outlined in the Teller Acuity Card Handbook. The cards were presented to the infants in stepwise sequence from the coarsest to finest grating, commencing with 0.32 cycles/cm and progressing in 0.5 octave steps. Acuity was recorded as the finest grating that the infant could repeatedly and reliably resolve, as judged by the tester based on the looking behavior of the infant. Confidence ratings, indicative of the tester's confidence in their assessment of each infant's acuity, were recorded using a 5-point scale in which 1 was lowest. The infants were tested at a distance of 38 cm under controlled lighting conditions by trained testers. In order to standardize the testing procedure, all the testers from all the sites were trained by author VD during the same week using a standardized training and testing procedure.

Analytical methods. Plasma and erythrocyte lipids were extracted, plasma phospholipid and erythrocyte phosphatidylcholine (PC) and phosphatidylethanolamine (PE) separated, and fatty acid analyses undertaken exactly as described elsewhere (12,14,27). The fatty acid composition of the milk samples was determined following preparation of methyl esters without solvent extraction (28) as in previous studies (12). Plasma total cholesterol and triglycerides, and apoA-I and B were analyzed using enzymatic, and specific immunoassays, respectively (29,30).

Data analysis. Looking acuity scores in cycles/cm were converted to cycles per degree, and a $\log_2$ transformation was applied to the data prior to statistical analyses. The vanElteren test, which is a nonparametric test, was used to detect any statistical significance among the groups (31). Two-way analyses of variance were used to determine the effect of age (14 d compared to 3 mon) and diet (breast-feeding compared to formula feeding) on plasma and erythrocyte fatty acids, plasma cholesterol, and growth. Formal tests of differences between diets at each age and between ages within a diet group were based on least-square means and standard errors calculated from SAS statistical software (Version 6; SAS Institute, Cary, NC).

RESULTS

Two hundred thirty-eight infants were enrolled in this multicenter study, with 24, 39, 10, 43, 54, 33, and 35 infants enrolled at the seven different centers. There were 99, 69, and 70 infants in the breast-fed, Formula 1, and Formula 2 groups, respectively. Of these, 191 successfully completed the protocol with 75, 59, and 57 in each of the same groups, respectively. Of the infants in the breast-fed group, 56 had no documented incidence of feeding with formula. The results for analyses of blood lipids, fatty acids, and visual acuity given for the breast-fed infants are for this group only. No significant differences in body weight, length, or head circumference which could be attributed to breast feeding or formula feeding were found at 3 mon (Table 2), or at 1 or 2 mon of age (data not shown). There were no statistically significant differences in body weight or growth among the different study centers for any of the diet groups.

TABLE 2
Indices of Growth Among Term Gestation Infants Who Were Breast-Fed or Fed Formula to 90 Days of Age[a]

	Weight (g)	Length (cm)	Head circumference (cm)
Birth			
Breast-fed (n = 56)	3466 ± 64	51.6 ± 0.3	35.1 ± 0.2
Formula 1 (n = 59)	3540 ± 61[b]	52.2 ± 0.3	34.8 ± 0.2
Formula 2 (n = 57)	3326 ± 62	51.6 ± 0.3	34.5 ± 0.2
90 d			
Breast-fed (n = 56)	6120 ± 98	61.3 ± 0.3	41.0 ± 0.2
Formula 1 (n = 59)	6110 ± 93	61.2 ± 0.3	40.7 ± 0.2
Formula 2 (n = 57)	5984 ± 95	60.5 ± 0.3	40.8 ± 0.2

[a]Values are means ± SEM. There were no statistically significant differences in the indices of growth among the diet groups at 90 d of age.
[b]At birth, infants in the group randomized to receive Formula 1 were significantly heavier than those in the group randomized to receive Formula 2, P = 0.046.

TABLE 3
Plasma Cholesterol, Triglycerides, Apo A-1, and Apo B of Term Infants Fed Human Milk or Formula at 14 and 90 d of Age[a]

	Breast-fed	Formula 1	Formula 2
14 d			
Cholesterol (mmol/L)	3.20 ± 0.06 (n = 52)	2.58 ± 0.06[b] (n = 54)	2.56 ± 0.06[b] (n = 53)
Triglyceride (mmol/L)	0.98 ± 0.10 (n = 52)	1.21 ± 0.08 (n = 54)	1.17 ± 0.09 (n = 53)
Apo A-1 (g/L)	1.24 ± 0.03 (n = 49)	1.24 ± 0.03 (n = 53)	1.23 ± 09.03 (n = 51)
Apo B (g/L)	0.48 ± 0.02 (n = 48)	0.30 ± 0.01[b] (n = 53)	0.31 ± 0.02[b] (n = 52)
90 d			
Cholesterol (mmol/L)	3.60 ± 0.07 (n = 54)	2.98 ± 0.06[b] (n = 56)	2.83 ± 0.06[b] (n = 57)
Triglyceride (mmol/L)	1.56 ± 0.10 (n = 54)	1.63 ± 0.08 (n = 56)	1.35 ± 0.08[c] (n = 57)
Apo A-1 (g/L)	1.40 ± 0.03 (n = 51)	1.32 ± 0.03 (n = 54)	1.29 ± 0.03[d] (n = 54)
Apo B (g/L)	0.62 ± 0.02 (n = 51)	0.41 ± 0.01[b] (n = 54)	0.40 ± 0.01[b] (n = 56)

[a]Values are means ± SEM. The statistical analyses utilized repeated measures of analysis of variance. The analyses shown were derived from comparing the groups at 14 and 90 d from the diet group age term.
[b]Significantly different from value for the breast-fed group, $P < 0.0001$.
[c]Significantly different from value for infants fed Formula 1, $P < 0.05$.
[d]Significantly different from value for the breast-fed group, $P = 0.008$.

The fatty acid composition of the breast milk (Table 1) was similar to that reported for other North American and European women (11). On day 90 postpartum, the milk fatty acids had (mean ± SD) 14.6 ± 4.2% LA, 1.2 ± 0.6% LNA, 0.5 ± 0.1% AA, and 0.2 ± 0.1% DHA. Levels of LNA were significantly higher, but AA and DHA, as well as 20:2n-6, 20:3n-6 and 22:4n-6 were significantly lower in milk collected at 90 d compared to 14 d postpartum. In comparison to the human milk, Formula 1 had 18.0% and 1.9% and Formula 2 had 34.2% and 4.7% LA and LNA, respectively, with no detectable AA or DHA in either product (Table 1).

At both 14 and 90 d of age, the breast-fed infants had significantly higher plasma concentrations of total cholesterol and apo B ($P < 0.0001$) but similar concentrations of triglycerides to the infants fed formula (Table 3). The plasma concentrations of apo A-I were significantly lower in the 90-d-old infants fed Formula 2 ($P = 0.0008$), but not in infants fed Formula 1 ($P = 0.054$), than in the breast-fed infants. There were no significant differences in the plasma cholesterol, apo A-I, or apo B concentrations between infants fed Formula 1 and infants fed Formula 2 at either 14 or 90 d of age. Infants fed Formula 2, however, had a lower ($P = 0.014$) plasma triglyceride concentration than infants fed Formula 1 at 90, but not at 14 d of age.

The composition of n-6 and n-3, as well as the saturated and monounsaturated fatty acids in the infant plasma phospholipids, and erythrocyte PC and PE at 90 d of age are given in Tables 4, 5, and 6, respectively. Only results for breast-fed infants who were not given any formula are included.

Compared with the breast-fed infants, the groups of infants fed Formula 1 and Formula 2 both had significantly higher levels of 18:2n-6, and significantly lower levels of 20:3n-6, 20:4n-6, 20:5n-3, and 22:5n-3 and 22:6n-3 in plasma phospholipids (Table 4) and erythrocyte PC (Table 5), and higher 18:2n-6, 20:2n-6 and 20:3n-6, but lower 20:4n-6, 20:5n-3, and 22:6n-3 in erythrocyte PE (Table 6). Infants fed Formula 2 also had a significantly higher percentage 20:2n-6, 18:3n-3, and 18:0 and lower 18:1 in plasma phospholipids and erythrocyte PC and PE than the breast-fed infants. In contrast to infants fed Formula 2, infants fed Formula 1 had a significantly higher 16:0 and 18:1 and lower 18:0 in plasma phospholipid, higher 16:0 and lower 18:0 in erythrocyte PC, and higher 18:1 and lower 22:5n-3 in erythrocyte PE than the breast-fed infants. The lower plasma and red blood cell (RBC) phospholipid levels of AA in the infants fed formula than in the breast-fed infants was not associated with lower body weight at 90 d of age; neither were there any significant relations between plasma phospholipid (Fig. 1) or RBC, PC or PE (data not shown), AA and growth or birthweight in the group of infants as a whole, or in the individual diet groups.

The amounts of LA and LNA in the formula clearly resulted in differences in the levels of LA (18:2n-6) and LNA (18:3n-3), as well as the longer chain n-6 and n-3 fatty acids in plasma and erythrocyte lipids between the two groups of formula-fed infants. As expected, there were no statistically significant differences in the infant plasma or RBC phospholipid fatty acids among the different study centers. Infants fed Formula 2, which was higher in LA and LNA than Formula 1, had significantly higher levels of 18:2n-6 and 18:3n-3 as well as 20:5n-3, 22:5n-3 and 22:6n-3, and lower 20:3n-6 and

TABLE 4
Major Fatty Acids in Plasma Phospholipids of Term Infants Fed Human Milk or Formula at 90 d of Age[a]

Fatty acid	Breast-fed (n = 54)	Formula 1 (n = 58)	Formula 2 (n = 57)
18:2n-6	22.8 ± 0.6	28.7 ± 0.5[b]	34.3 ± 0.5[b,c]
20:2n-6	0.4 ± 0.0	0.3 ± 0.0	0.6 ± 0.0[b,c]
20:3n-6	2.8 ± 0.1	2.2 ± 0.1[b]	1.8 ± 0.1[b,c]
20:4n-6	12.2 ± 0.3	6.6 ± 0.2[b]	6.8 ± 0.3[b]
22:4n-6	0.5 ± 0.0	0.4 ± 0.0[b]	0.4 ± 0.0[b]
22:5n-6	0.5 ± 0.0	0.4 ± 0.0[b]	0.2 ± 0.0[b,c]
18:3n-3	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0[b,c]
20:5n-3	0.3 ± 0.0	0.1 ± 0.0[b]	0.2 ± 0.0[b,c]
22:5n-3	0.9 ± 0.0	0.4 ± 0.0[b]	0.6 ± 0.09[b,c]
22:6n-3	3.8 ± 0.1	1.4 ± 0.1[b]	1.7 ± 0.1[b,c]
16:0	23.9 ± 0.3	26.6 ± 0.3[b]	23.5 ± 0.3[c]
18:0	17.8 ± 0.3	16.8 ± 0.3[b]	19.9 ± 0.3[b,c]
18:1	12.2 ± 0.4	13.9 ± 0.4[b]	7.8 ± 0.4[b,c]

[a]Values are means ± SEM in weight percentage total fatty acids. The statistical analyses utilized repeated measures of analysis of variance. The analyses shown were derived from comparing the groups at 90 d from the diet age term.
[b]Significantly different from value for infants fed human milk, $P < 0.05$.
[c]Significantly different from value for infants fed Formula 1, $P < 0.05$.

22:5n-6 in plasma phospholipids (Table 4), higher 18:2n-6, 18:3n-3, 20:2n-6, 20:5n-3 and 22:5n-3, and lower 20:3n-6 and 22:5n-6 in erythrocyte PC (Table 5), and higher 18:2n-6, 20:2n-6, 18:3n-3, 20:5n-3 and 22:5n-3 and lower 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6 in erythrocyte PE (Table 6) than infants fed Formula 1. Presumably, the higher levels of 18:3n-3, 20:5n-3, and 22:5n-3 in the blood lipids of infants fed Formula 2 than in infants fed Formula 1 reflects the difference in the level of 18:3n-3 in the two formula (percentage 18:3n-3, 1.9% and 4.7% for Formulae 1 and 2, respectively).

The differences in composition of saturated and monounsaturated fatty acids in the formulae also had a significant effect on the plasma and erythrocyte phospholipid fatty acids of the formula-fed infants. Infants fed Formula 1, which had higher levels of 16:0 and 18:1 than Formula 2, had significantly higher 16:0 and 18:1 and lower 18:0 in plasma phos-

TABLE 5
Major Fatty Acids in Erythrocyte Phosphatidylcholine of Term Infants Fed Human Milk or Formula at 90 d of Age[a]

Fatty acid	Breast-fed (n = 55)	Formula 1 (n = 58)	Formula 2 (n = 57)
18:2n-6	18.5 ± 09.4	23.0 ± 0.4[b]	27.3 ± 0.4[b,c]
20:2n-6	0.5 ± 0.0	0.5 ± 0.0	0.8 ± 0.0[b,c]
20:3n-6	2.0 ± 0.1	1.6 ± 0.1[b]	1.2 ± 0.1[b,c]
20:4n-6	7.7 ± 0.3	4.5 ± 0.3	4.4 ± 0.3[b]
22:4n-6	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
22:5n-6	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0[b,c]
18:3n-3	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.0[b,c]
20:5n-3	0.2 ± 0.0	0.1 ± 0.0[b]	0.1 ± 0.0[b,c]
22:5n-3	0.4 ± 0.0	0.2 ± 0.0[b]	0.3 ± 0.0[b,c]
22:6n-3	1.6 ± 0.1	0.6 ± 0.1[b]	0.7 ± 0.1[b]
16:0	31.8 ± 0.4	33.6 ± 0.4[b]	30.9 ± 0.4[c]
18:0	14.2 ± 0.2	13.0 ± 0.2[b]	16.1 ± 0.2[b,c]
18:1	20.1 ± 0.4	20.0 ± 0.4	15.2 ± 0.4[b,c]

[a]Values are means ± SEM in weight percentage total fatty acids. The statistical analyses utilized repeated measures of analysis of variance. The analyses shown were derived from comparing the groups at 90 d from the diet group age term.
[b]Significantly different from value for infants fed human milk, $P < 0.05$.
[c]Significantly different from value for infants fed Formula 1, $P < 0.05$.

TABLE 6
Major Fatty Acids in Erythrocyte Phosphatidylcholine of Term Infants Fed Human Milk or Formula at 90 d of Age[a]

Fatty acid	Breast-fed (n = 55)	Formula 1 (n = 58)	Formula 2 (n = 57)
18:2n-6	6.7 ± 0.3	9.0 ± 0.3[b]	12.0 ± 0.3[b,c]
20:2n-6	0.4 ± 0.0	0.5 ± 0.0[b]	0.9 ± 0.0[b,c]
20:3n-6	1.5 ± 0.0	2.0 ± 0.0[b]	1.8 ± 0.0[b,c]
20:4n-6	25.9 ± 0.6	23.9 ± 0.2[b]	22.2 ± 0.5[b,c]
22:4n-6	8.2 ± 0.2	8.7 ± 0.2	7.8 ± 0.2[c]
22:5n-6	1.5 ± 0.1	1.5 ± 0.1	1.1 ± 0.1[b,c]
18:3n-3	0.3 ± 0.0	0.4 ± 0.0	0.6 ± 0.0[b,c]
20:5n-3	0.5 ± 0.0	0.3 ± 0.0[b]	0.4 ± 0.0[b,c]
22:5n-3	2.9 ± 0.1	2.0 ± 0.1[b]	2.9 ± 0.1[c]
22:6n-3	6.9 ± 0.2	4.0 ± 0.2[b]	4.0 ± 0.2[b]
16:0	14.3 ± 0.3	14.9 ± 0.3	15.4 ± 0.3[b]
18:0	8.1 ± 0.3	8.1 ± 0.2	9.9 ± 0.2[b,c]
18:1	20.3 ± 0.5	22.0 ± 0.4[b]	18.6 ± 0.4[b,c]

[a]Values are means ± SEM in weight percentage total fatty acids. The statistical analyses utilized repeated measures of analysis of variance. The analyses shown were derived from comparing the groups at 90 d from the diet group age term.
[b]Significantly different from value for infants fed human milk, $P < 0.05$.
[c]Significantly different from value for infants fed Formula 1, $P < 0.05$.

pholipids and erythrocyte PC, and lower 18:0 and higher 18:1 in erythrocyte PE than infants fed Formula 2. Although the 16:0 and 18:1 levels in Formula 1 were similar to those in the human milk (Table 1), the plasma phospholipid 16:0 and 18:1, the erythrocyte PC 16:0, and the erythrocyte PE 18:1 were significantly higher in infants fed Formula 1 than in the breast-fed infants.

There were no significant differences in preferential look-

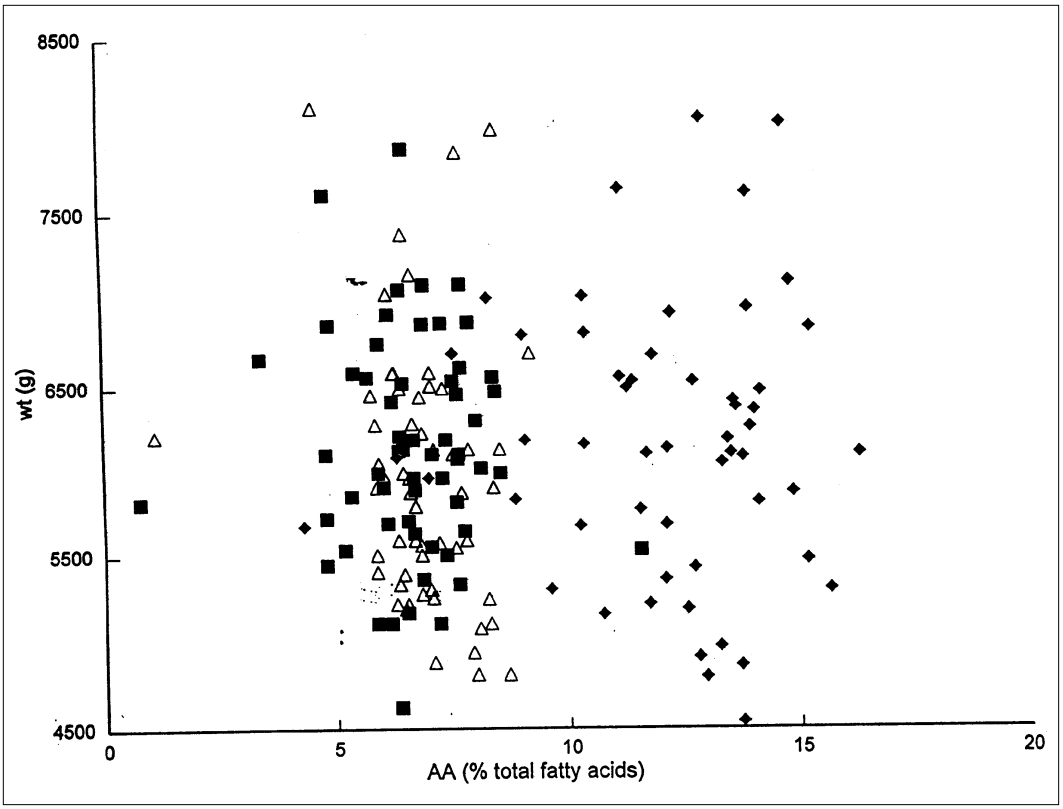

FIG. 1. Plasma phospholipid arachidonic acid (AA) and weight of 90-day-old infants who were breast-fed with no formula supplementation (◆), or fed formula 1 (■) or formula 2 (△). No statistically significant relations were found either for the group as a whole or for the individual diet groups.

TABLE 7
Preferential Looking Acuity in 90-Day-Old Term Infants Fed Human Milk or Formula[a]

Fatty acid	Breast-fed[b] (n = 56)	Formula 1 (n = 59)	Formula 2 (n = 57)
Acuity	2.79 ± 0.77	2.51 ± 0.50	2.67 ± 0.64
Range	0.22–5.53	1.11–4.49	1.11–6.77

[a]Values are given as means in cycles/degrees ± SD in octaves, with the range of acuity in cycles/degree. The statistical analyses utilized repeated measures of analysis of variance. The analyses shown were derived from comparing the groups at 90 d from the diet group age term.
[b]Infants who were breast-fed with no formula supplementation. There were no statistically significant differences among the groups.

ing acuity among the 90-day-old breast-fed and formula-fed infants, when comparisons were made with either the entire group of breast-fed infants (n = 75, P = 0.071) or the group of breast-fed infants (n = 56, P = 0.089) who received no formula supplementation (Table 7). Further, no significant differences in visual acuity scores were found among the study centers for the different groups. The visual acuity for all infants in the human milk group (n = 75) was 2.81 cycle/degrees ± 0.69 octaves, and was not different from the acuity results of the other groups. One infant in the group of breast-fed infants had a visual acuity of 0.22 cycles/degree, the next lowest acuity recorded for infants in this group was 1.11 cycles/degree. Removal of the result for this infant did not change the results of statistical analysis. A poststudy power analysis was done to ensure this study had sufficient power to detect a clinically relevant difference of 0.5 octaves in visual acuity between the groups, with an alpha level of 0.05. These calculations showed that this study had a power of at least 97% to detect a 0.5 octave difference in visual acuity among the groups. Tester confidence ratings for the 56, 59, and 57 infants who were breast-fed only, or fed Formula 1 or 2, were: 5 points, 43, 41, 40, 4 points, 13, 16, 15, and 3 points, 0, 2, 2 respectively. Confidence ratings of 1 or 2 were not recorded for any visual acuity test. One of the potential problems with a multicenter study is the possibility of variability in the infant populations at the different study centers. This study found no statistically significant differences in visual acuity scores among infants from the different centers. Analyses were also undertaken to explore potential relations between visual acuity and DHA in the plasma and erythrocyte PC (data not shown) and erythrocyte PE (Fig. 2). No statistically significant relations were found between DHA in any lipid fraction analyzed and visual acuity either for the group of infants as a whole or within the groups of breast-fed (with or without formula supplemented) or formula-fed infants.

DISCUSSION

This multicenter, prospective study found no difference in growth or in preferential looking acuity at 90 d of age among breast-fed infants and infants fed a formula with 18.0% LA (18:2n-6) and 1.9% LNA (18:3n-3), or 34.2% LA and 4.7% LNA with no AA or DHA. The looking acuities found here are well within recently reported binocular tolerance limits for visual acuity at 3 mon of age as measured using the acuity card procedure (24). The findings of these studies are also consistent with a large cross-sectional study of over 400 9-mon-old infants (17), as well as with other prospective studies that show similar preferential looking acuity in healthy term gestation breast-fed infants and infants fed formula with about 18% LA and 2.0% LNA with no AA or DHA from birth to 90 d (12), or to 1 year of age (16). In the latter study, visual evoked potential (VEP) and scores in developmental tests were also similar between breast-fed infants and infants fed formula without AA or DHA when measured from birth to 14 months of age (16,32). Another study using similar formula, however, found lower looking acuity in formula-fed than breast-fed infants at 2 but not at 4 mon of age (18). Studies by Jorgensen *et al.* (19) in Denmark also reported a higher increase in looking acuity from 2 to 4 mon of age in a group of breast-fed compared to formula-fed infants and similarly, studies in Australia have found a lower VEP acuity at 5 mon of age in a group of 8 formula-fed infants than in 8 breast-fed infants (13). Another recent study from Australia described a lower mean acuity, when extrapolated from VEP data, at 16 and 30 wk of age in infants fed formula with about 16.8% LA and 1.6% LNA than in breast-fed infants. Infants fed a formula with 0.3% 18:3n-6, 0.5% 22:5n-3, and 0.4% 22:6n-3 from evening primrose oil and fish oil had VEP acuity results which were not significantly different from the group of breast-fed infants. It is not clear if differences in the results among studies are explained by differences in LNA content, LA/LNA ratio, or other nutrients in the formulae, or by other socioeconomic or environmental differences in the groups of nonrandomized breast-fed and formula-fed infants. Differences in the methodologies used to assess visual function could also potentially explain some of the discrepancies in the findings. However, although acuity is higher when measured with electrophysiological methods, there is a close correlation between visual acuity measured with acuity cards and both sweep and steady-state VEP in infants (33).

An important feature of the study reported here which could influence the ability to detect significant group differences in visual acuity was the use of a multicenter design. Two aspects of the acuity card procedure, however, make it particularly suitable for multicenter studies. First, test duration is short, with an average test duration of 3–5 min, and the equipment and procedures are well standardized. Second, al-

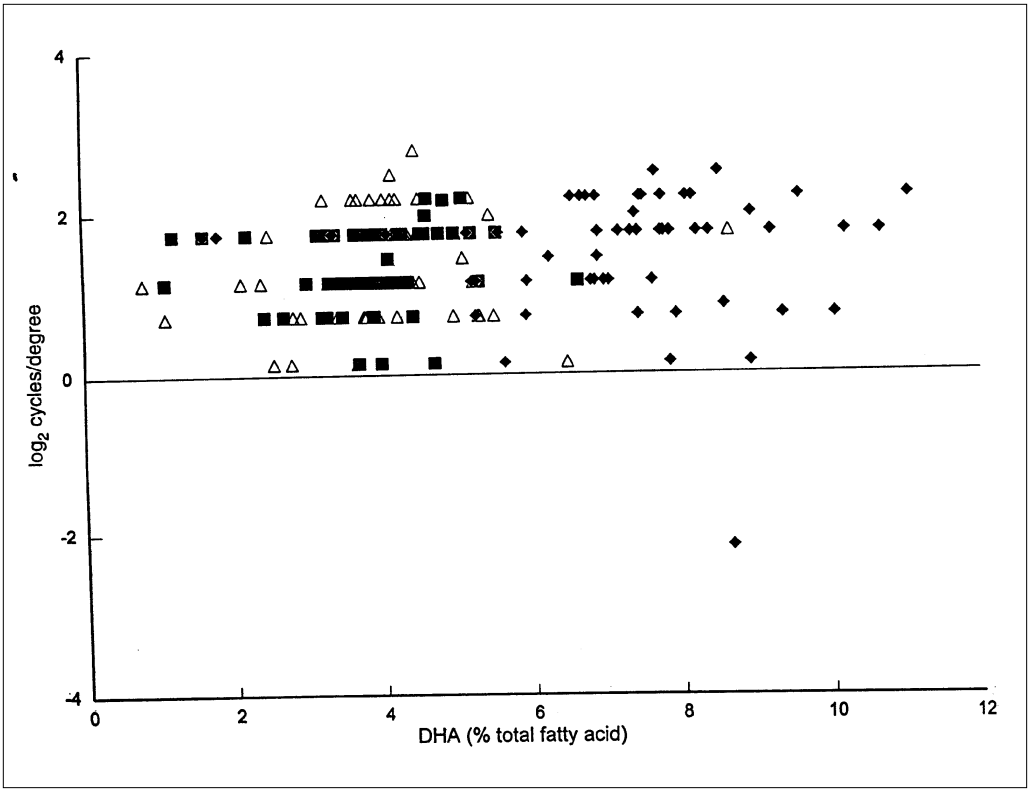

FIG. 2. Erythrocyte phosphatidylethanolamine docosahexaenoic acid (DHA) and visual acuity in $\log_2$ cycles/degree of 90-day-old infants who were breast-fed with no formula supplementation (◆), or fed formula 1 (■) or formula 2 (△). No statistically significant relation between DHA and visual acuity is present for the group as a whole, or for the individual diet groups.

though the testing procedure requires training and practice, no particular technical skills are needed (34). For this study, group training and practice was provided for all of the testers at a single center. A limitation of the methodology, however, is that it was not possible to obtain measures of intertester reliability. Genetic and/or environmental variables among subjects and differences in the test environment among the centers may also decrease the power to find differences due to diet.

Evidence for an inverse relation between blood lipid AA and growth in premature infants has been reported (21), and a recent preliminary report described lower growth among infants fed formula with 4% compared to 1% LNA (22). In the study reported here, the growth of healthy term infants fed formula was not different from that of breast-fed babies, despite the two-fold difference in plasma phospholipid AA level between the breast-fed and formula-fed infants. Further, no effect of the formula content of LNA (4.7% compared to 1.9%), or palmitic acid (16:0), oleic acid (18:1) or LA/LNA ratios of 9.5 and 7.3 on growth was found. Consistent with these results, other studies have also found that term gestation infants fed formula grow at least as well as breast-fed infants (12,14,23,35).

Dietary polyunsaturated and monounsaturated fatty acids result in lower plasma total and LDL cholesterol than saturated fatty acids, particularly 14:0 and 16:0 (36,37). Polyunsaturated, but not monounsaturated fatty acids, however, can also lower high density lipoprotein (HDL) cholesterol. The studies reported here included analyses of apo A-I and apo B, which are the major apoprotein components of HDL and LDL, respectively, in infants fed formulae which differed in saturated, monounsaturated, and polyunsaturated fatty acids and in breast-fed infants. As shown by others (35,38,39), the plasma total cholesterol and apo B concentrations in infants fed formula was uniformly lower than in breast-fed infants. Despite a two-fold difference in the 16:0 content of the formulae (22.2 and 10.3% 16:0), neither the plasma total cholesterol nor the apo B concentration differed between the groups of formula-fed infants. Infants fed the formula which was high in polyunsaturated fatty acids (Formula 2, 34.2% LA and 4.7% LNA) did have significantly lower plasma apo A-I levels than infants who were breast-fed. This finding is consistent with information that shows polyunsaturated, but not monounsaturated, fatty acids may lower plasma HDL (36). Whether these differences in plasma lipoproteins are of any physiological significance to the young infant is not yet clear. Possibly, the lack of an effect of the differences in dietary saturated fatty acid intake on plasma cholesterol in the formula-fed infants in these studies is explained by the absence of significant amounts of cholesterol in liquid formu-

lae. In this regard, although studies by van Biervliet *et al.* show that addition of cholesterol to formula can increase plasma cholesterol in formula-fed infants, Hayes and Khosla (40) have hypothesized that dietary 16:0 does not raise serum cholesterol when the cholesterol intake is very low.

As is now well known (12–15), and again shown here, infants fed formula without AA or DHA have lower blood lipid levels of AA and DHA than breast-fed infants. In the study reported here, infants fed a formula with 4.7% LNA in a LA/LNA ratio of about 7.3:1 had modestly but significantly higher plasma phospholipid levels of DHA than infants fed a formula with 1.9% LNA, in a LA/LNA ratio of about 9.5:1. The difference in DHA between infants fed the two formulae was accompanied by an inverse change in the plasma phospholipid 22:5n-6. A similar difference in 22:5n-6 was present in both the erythrocyte PC and PE. Previous studies have found lower erythrocyte PC and PE 22:5n-6 and higher PE DHA in 4.5–6-month-old term infants fed formula with 45.1% LA and 5.0% LNA than in infants fed formula with 14.0% LA and 1.2% LNA (15). These findings suggest that relatively high compared to lower amounts of LA in formula (up to 34% fatty acids) do not limit the conversion of LNA to DHA at least when provided in LA/LNA ratios under 10:1. It also seems that higher amounts of LA in formulae do not result in preferential synthesis of AA or its elongation and further desaturation. For example, Formula 1 with 18% LA resulted in a significantly higher infant erythrocyte PE AA than Formula 2 which had 34.2% LA. Presumably, the lower levels of 22:5n-6 found in association with higher LA in formulae in this and previous studies (15) are explained by the accompanying higher amount of LNA resulting in inhibition of n-6 fatty acid desaturation. This suggestion is consistent with the inverse relationship between plasma and tissue levels of 22:5n-6 and the dietary LNA supply (2).

Providing levels of 16:0 and 18:1 in formulae similar to the mean levels in human milk of about 21 and 36% total fatty acids, respectively, resulted in plasma phospholipid levels of 18:0 and 18:1 in formula-fed infants closer to those of breast-fed infants. The significance of differences in plasma saturated and monounsaturated fatty acids in young infants is not known, but in young piglets these differences are accompanied by differences in the saturated and monounsaturated fatty acid composition of liver phospholipids (41). Thus, it seems reasonable to assume that formulae should provide a balance of monounsaturated fatty acids which result in blood lipid monounsaturated fatty acid levels similar to those of breast-fed infants.

In summary, the results of this multicenter trial show no measurable differences in visual acuity, as measured with the acuity card procedure, or growth among 90-day-old infants fed formula with 1.9% or 4.7% LNA and no DHA or AA, and breast-fed infants. Continued study of the dietary requirement for various n-6 and n-3 fatty acids and of their role in infant development is clearly needed to resolve the different findings from others working on this issue.

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Synthesis of a Novel Vitamin E Derivative, 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol, by α-Glucosidase-Catalyzed Transglycosylation

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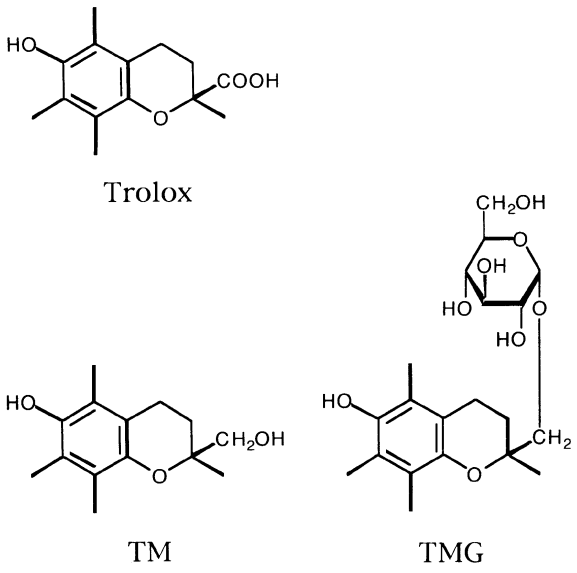
ABSTRACT: A novel derivative of vitamin E, vitamin E glucoside, was synthesized from 2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol and maltose in a solution containing DMSO by transglycosylation with α-glucosidase from *Saccharomyces* species. The glycosylated product was identified as 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG) by mass spectrometry and nuclear magnetic resonance spectroscopy. The optimal pH of transglycosylation was 5.5, and the yield of TMG increased as the concentration of maltose increased. TMG has high solubility in water (>1×10^3 mg/mL). The 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity of TMG was found to be nearly the same as those of α-tocopherol, Trolox (2-carboxy-2,5,7,8-tetramethylchroman-6-ol), and ascorbic acid.

Lipids 31, 73–78 (1996).

Many of the most serious human diseases, including cancer and heart disease, at some stage involve oxidation processes mediated by free radicals (1). Much attention has been focused on the protective effect by antioxidants against damage caused by active oxygen and free radicals *in vivo*. There has been considerable interest in the effectiveness of antioxidants in the therapy and prevention of human disease. Among the antioxidants, the effectiveness of vitamin E, a chain-breaking antioxidant, is well known (2–6). Vitamin E is a lipophilic compound, and it is generally accepted that it is incorporated into biological membranes and is especially effective as a lipid-peroxyl radical scavenger (4,7,8). Vitamin E also scavenges active oxygen species such as superoxide anion radical (9), singlet molecular oxygen (10), and hydroxyl radical (11). The antioxidant properties of vitamin E are based on the ability of the chromanoxyl ring to interact with different free radicals (3,12). Since the mobility of vitamin E in the biological membranes is restricted due to its long phytyl side chain, it seems to be unlikely to scavenge active oxygens which might be generated in aqueous phase. In recent studies, the significance of a water-soluble analog of vitamin E, Trolox (2-carboxy-2,5,7,8-tetramethylchroman-6-ol, Scheme 1), has been reported (13–17). In practice, however, Trolox is nearly insoluble in water. We believe that introduction of glucose into the molecule of vitamin E analog will increase its water solubility to scavenge reactive oxygen species in the aqueous phase efficiently.

Enzymatic transformation has been utilized to improve the chemical properties and physiological functions of biologically useful substances (18–20). α-Glucosidase (EC 3.2.1.20) has been found to catalyze transglycosylation (21,22). If the carboxyl group of Trolox is reduced to the corresponding alcohol, 2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol (TM), the chemical properties, and the solubility in water of



SCHEME 1

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Abbreviations: DMSO, dimethyl sulfoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FAB–MS, fast atom bombardment–mass spectrometry; HPLC, high-performance liquid chromatography; IR, infrared; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TM, 2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol; TMG, 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol; Trolox, 2-carboxy-2,5,7,8-tetramethylchroman-6-ol; UV, ultraviolet.

this vitamin E derivative might be easily modified by the transglycosylation reaction. In this paper, we describe the synthesis of a novel vitamin E glucoside, 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG), by α-glucosidase-catalyzed transglycosylation using an aqueous–organic solvent system. The water-solubility and free-radical scavenging activity of TMG are also described.

EXPERIMENTAL PROCEDURES

Materials. (*R,S*)-Trolox and its optically active isomers, (*R*)- and (*S*)-forms, were obtained from Aldrich Chemical Co. (Milwaukee, WI). *RRR*-α-Tocopherol was obtained from Eisai Co. (Tokyo, Japan). α-Glucosidase from *Saccharomyces* sp. (Diagnostic Reagent Grade) and β-glucosidase from sweet almond (Diagnostic Reagent Grade) were obtained from Toyobo Co. (Osaka, Japan). Centrifugal ultrafiltration tubes (Ultrafree MC-LG, 0.2 mm pore size) were purchased from Millipore (Milford, MA). All other reagents were of analytical grade.

Preparation of TM. Trolox was reduced with $LiAlH_4$ to produce the corresponding TM. To a solution of (*R,S*)-Trolox (2.0 g) in diethyl ether (200 mL) was added 1.0 g of $LiAlH_4$. The suspension was refluxed for 20 h. After cooling on an ice bath, the reaction was stopped by adding 15 mL of concentrated HCl. The diethyl ether phase was washed with water, dried over anhydrous Na_2SO_4, and the solvent was removed *in vacuo*. The resulting pale-yellow powder of TM was purified by silica-gel column chromatography with hexane/ethyl acetate (1:1, vol/vol) as the solvent. The elution was checked by thin-layer chromatography (TLC) on a precoated silica gel 60 plate (E. Merck, Darmstadt, Germany) using hexane/diethyl ether/acetic acid (50:60:1, by vol). The TM fraction that gave a single spot (R_f 0.69) on the TLC plate was combined, passed through a 0.45-μm filter, and the solvent was removed *in vacuo*. Pure (*R,S*)-TM was thus obtained as a white powder (1.8 g yield) (23,24): infrared (IR) (KBr) ν_{max} 3400, 2920, 1640, 1460, 1420, 1380, 1260, 1150, 1110, 1085, 1060, 1050, and 920 cm^{-1}; ^1H NMR dimethyl sulfoxide [(DMSO)-d_6] δ 1.14 (*s*, 3H), 1.60–1.88 (*m*, 2H), 1.98 (*s*, 3H), 2.02 (*s*, 3H), 2.05 (*s*, 3H), 2.51 (broad *t*, 2H), 3.35 (*q*, *J* = 10.7 Hz, 2H), 4.78 (broad *s*, 1H), and 7.38 (broad *s*, 1H). The (*R*)- and (*S*)-forms of TM were also prepared from (*R*)- and (*S*)-forms of Trolox, respectively.

Glucosidase activity. The α-glucosidase activity was determined by measuring the amount of glucose formed. The reaction mixture contained 100 μL of 4% maltose solution, 300 μL of 0.1 M potassium phosphate buffer (pH 6.5), and 40 μL of enzyme solution. After the mixture was incubated at 37°C for 20 min, the reaction was stopped by heating in a boiling water bath for 5 min. The glucose content was determined by the glucose oxidase method (25) using a glucose assay kit (Wako Pure Chemical Industries, Osaka, Japan). One unit of α-glucosidase activity was defined as the amount of enzyme which hydrolyzed 1 μmol of maltose per min.

Enzyme reaction. The enzyme reaction was carried out in an aqueous–organic solvent system using DMSO as the organic solvent. The standard reaction mixture contained 1.0 mL of 40% maltose in 50 mM potassium phosphate buffer (pH 6.0), 200 μL of 5% TM in DMSO, and 50 μL of α-glucosidase (5 U) in 50 mM potassium phosphate buffer (pH 6.5). The reaction was carried out at 40°C for 20 h. An aliquot (200 μL) sample was withdrawn, dissolved in 1.0 mL of acetonitrile/water (1:1, vol/vol), and analyzed by TLC and high-performance liquid chromatography (HPLC).

HPLC. HPLC was done with a Hitachi L-6300 pump equipped with a L-4250 ultraviolet (UV)-visible and a L-3350 refractive index detectors (Hitachi Co., Tokyo Japan). TM and its glucoside, TMG, in the reaction mixture were analyzed by an Inertsil ODS-2 column (6.0 × 150 mm; GL Sciences Inc., Tokyo, Japan) developed with acetonitrile/water (40:60, vol/vol) at a flow rate of 1.0 mL/min. The eluent was monitored by an absorbance at 280 nm. The *R/S* ratio of TMG was analyzed by a Wakosil-II 5C18 HG column (4.6 × 250 mm; Wako Pure Chemical Industries) developed with acetonitrile/water/10% H_3PO_4 (20:80:0.1, by vol) at a flow rate of 0.5 mL/min. Maltose and glucose were analyzed by a Shodex Asahipak NH2P-50 column (4.6 × 250 mm; Showa Denko, Tokyo, Japan) with acetonitrile water (75:25, vol/vol) at 1.0 mL/min. The eluent was monitored by a refractive index.

Spectral analysis. UV spectra were measured with a Hitachi U-3210 spectrophotometer. IR spectra were measured on potassium bromide pellets using a Jasco FT/IR-8000 IR spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan). Fast atom bombardment–mass spectrometry (FAB–MS) was done with a Shimadzu 9020-DF instrument (Shimadzu Co., Kyoto, Japan) with a xenon fast atom bombardment gun. Samples were placed in the 5.5 kV xenon atom beam on a probe with glycerol as the matrix. ^1H (270.17 MHz) and ^{13}C (67.9 MHz) nuclear magnetic resonance (NMR) spectra were recorded at 25°C on a Jeol GX-270 FT-NMR spectrometer (Jeol, Tokyo, Japan) using DMSO-d_6 as solvent and tetramethylsilane as internal standard. Specific rotations were determined with a Union PM-201 automatic digital polarimeter (Union Gikken, Osaka, Japan).

Preparation of TMG. The reaction mixture contained 80 mL of 60% maltose in 50 mM potassium phosphate buffer (pH 6.0), 16 mL of 5% TM in DMSO, and 0.1 mL of α-glucosidase (400 U) in 50 mM potassium phosphate buffer (pH 6.5). After the reaction was carried out at 40°C for 20 h, the reaction mixture was applied on an Amberlite XAD-4 column (4 × 40 cm) equilibrated with 30% methanol. The column was washed with the same solvent to remove DMSO and sugars. TMG and TM were then eluted with 80% methanol. The eluent was checked by TLC using chloroform/methanol/acetic acid/water (40:10:2:1, by vol). Compounds were visualized by spraying with H_2SO_4/methanol (1:1, vol/vol) followed by charring. Spots with reducing power were detected by spraying with the ferric chloride/bathophenanthroline reagent (26). The TMG fraction was combined, and the solvent was removed *in vacuo*. The resulting white powder was further pu-

rified by silica-gel column chromatography using ethyl acetate/methanol (5:1, vol/vol). Pure TMG was obtained as a white powder (200 mg yield).

Enzymatic hydrolysis of TMG. The reaction mixture containing 1.0 mL of 5 mM TMG in 50 mM potassium phosphate buffer (pH 6.0) and 50 μL of a glucosidase (5 U, α-glucosidase from *Saccharomyces* sp. or β-glucosidase from sweet almond), incubated at 40°C for 3 h. An aliquot (200 μL) was withdrawn and mixed with 1.0 mL of acetonitrile/water (1:1, vol/vol). The TM and TMG were analyzed by HPLC. The liberated glucose was analyzed by the glucose oxidase method (25).

Solubility of vitamin E analogs. Each vitamin E analog (300 mg) was put into a 3 mL glass vial, after which 250 μL of water was added. The mixture was placed on a water bath equipped with a magnetic stirrer, and was stirred at 200 rpm at 25°C for 20 h. The mixture was then transferred into a centrifugal ultrafiltration tube and centrifuged at 2000 × *g* for 10 min. The amount of each vitamin E analog in the filtrate was determined by revered-phase HPLC.

Scavenging of 1,1-diphenyl-2-picrylhydrozyl (DPPH). Free-radical scavenging activity of each antioxidant was assayed using a stable free radical, DPPH, according to the method of Blois (27). The reaction mixture contained 1 mL of 0.5 mM DPPH in ethanol and 4 mL of 0.1 mM antioxidant in ethanol/water (50:50, vol/vol). Finally, the total volume of the reaction mixture was adjusted to 5 mL with ethanol. After the reaction was carried out at room temperature for 20 min, the free-radical scavenging activity of each antioxidant was quantified by the decolorization of DPPH at 516 nm.

RESULTS

Transglycosylation of maltose to TM. α-Glucosidase from *Saccharomyces* sp. could catalyze the transglycosylation to TM from maltose to produce a novel vitamin E glucoside, TMG, in an aqueous DMSO solution. Figure 1 shows the α-glucosidase-catalyzed reaction product of TM with maltose after 20 h incubation. A peak corresponding to the glucoside of TM appeared at 3.3 min. The glucoside gave a red spot (R_f 0.40) on a TLC plate by the ferric chloride/bathophenanthroline reagent, indicating the presence of the reducing power (26).

A large amount of TMG was prepared from (*R,S*)-TM and maltose according to the procedure as described in the Materials and Methods section. TMG was hydrolyzed by α-glucosidase (the degree of hydrolyis was 80%) and produced an equimolar ratio of TM and glucose. On the other hand, β-glucosidase did not hydrolyze TMG. The FAB–MS of TMG showed a molecular ion at *m/z* 398 (M^+, 100%) in addition to other fragment ions: 237 (98), 219 (26), 205 (27), 191 (16), and 165 (75). Table 1 shows the ^{13}C NMR spectra of (*R,S*)-, (*R*)-, and (*S*)-forms of TMG and (*R,S*)-TM in DMSO-d_6. The ^{13}C chemical shifts of the chromanol moiety were essentially the same as those of TM except for the C-2 and C-2a shifts. The C-2a signal of TMG was shifted downfield (3.3 ppm) relative to that of TM, and the C-2 signal was shifted slightly upfield (1.0 ppm). The signals of C-2 carbon atom could be resolved by the data of optically active (*R*)- and (*S*)-forms of TMG. The remaining signals of TMG were assigned as the carbons of α-D-glucopyranosyl moiety. The α-configuration of the D-glucosyl residue in the TMG was confirmed by the C-1 signal at δ 98.8. From these data and other spectral data, the structure of TMG was identified as 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol: UV (ethanol) λ_{max} 291 nm (ε 3000); IR (KBr) ν_{max} 3400, 2920, 1640, 1460, 1420, 1380, 1260, 1150, 1110, 1030, and 920 cm^{-1}; and $[\alpha]_D^{25}$ +85.6 (c 0.500, ethanol). (*R*)-TMG, $[\alpha]_D^{25}$ +90.4 (c 0.500, ethanol); and (*S*)-TMG, $[\alpha]_D^{25}$ +79.6 (c 0.500, ethanol). The *R/S* ratio of TMG produced from (*R,S*)-TM was determined to be 1.3:1 by the HPLC analysis.

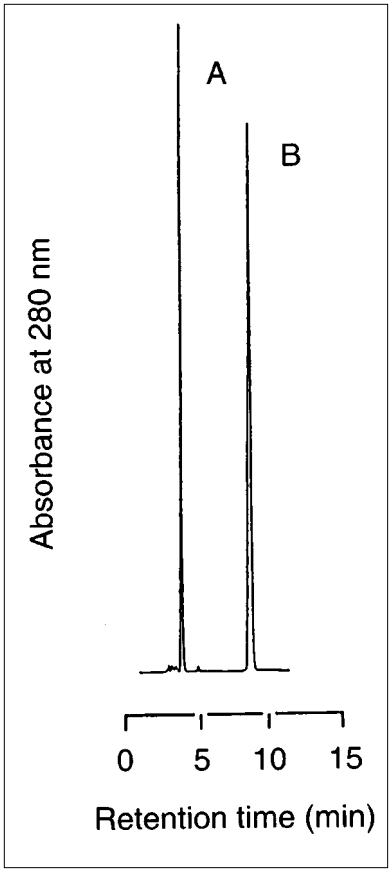


FIG. 1. Reversed-phase high-performance liquid chromatography (HPLC) of the transglycosylation products from maltose to TM by α-glucosidase. The reaction was carried out at 40°C for 20 h as described in the Experimental Procedures section. HPLC was done with an Inertsil ODS-2 column (4.6 × 150 mm) (GL Sciences, Inc., Tokyo, Japan) which was developed with acetonitrile/water (40:60, vol/vol) at 1.0 mL/min. Peak A, the transglycosylation product of TM; and peak B, TM. See Scheme 1 for other abbreviation.

Figure 2 shows the effect of pH on the hydrolysis of maltose and on the synthesis of TMG by α-glucosidase. While the optimal pH for hydrolysis of maltose was 7.0, the pH for synthesis of TMG was 5.5. On the other hand, the two reactions had the same optimal temperature to be at 40°C (data not shown). The effect of maltose concentration on the syn-

TABLE 1
^{13}C Nuclear Magnetic Resonance Chemical Shifts of (*R,S*)-, (*R*)-, and (*S*)-Forms of 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (TMG) and (*R,S*)-2-Hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol (TM)[a]

(*R,S*)-TMG	(*R*)-TMG	(*S*)-TMG	(*R,S*)-TM	Assignment[b]
				Chromanol moiety
73.8 and 73.9	73.9	73.8	74.8	2
70.0 and 70.1	70.1	70.0	66.8	2[a]
22.2 and 22.4	22.2	22.3	21.7	2[b]
28.2	28.2	28.2	28.0	3
19.7 and 19.8	19.7	19.8	19.9	4
116.65 and 116.72	116.65	116.69	116.9	4[a]
120.81 and 120.84	120.81	120.83	120.2	5
11.7	11.7	11.7	11.8	5[a]
144.2	144.2	144.2	144.6	6
120.17 and 120.21	120.21	120.15	121.0	7
12.6	12.6	12.6	12.7	7[a]
122.5	122.5	122.5	122.6	8
145.1	145.1	145.1	145.1	8[a]
11.7	11.7	11.7	11.8	8[b]
				Glucose moiety
98.80 and 98.85	98.85	98.77		1
72.6 and 72.9	72.9	72.6		2
73.1	73.1	73.1		3
71.2 and 71.5	71.5	71.1		4
71.9	71.9	71.9		5
60.6 and 60.8	60.8	60.6		6

[a]Shifts in parts per million downfield relative to tetramethylsilane.
[b]The numbering systems used are the standard numbering systems for the chromanol and glucose moieties.

thesis of TMG is shown in Figure 3. The yield of TMG increased as the concentration of maltose increased, and reached to maximal level at 60%. The effect of TM concentration on the synthesis of TMG is shown in Figure 4. TM is scarcely soluble in water, and the solubility of TM depends on the concentration of DMSO in the reaction mixture. To evaluate the optimal concentration of TM for the transglycosylation reaction, the volumetric ratios between the acceptor solution (as 5% TM in DMSO) and the donor solution (as 40% maltose in buffer) were changed. When the ratio between the two solutions was changed from 1:11 to 4:8 by volume, the amount of TMG formation increased as ratios of the TM solution until the ratio came to be 3:9. In contrast, the proportion of TM used for the production of TMG in the re-

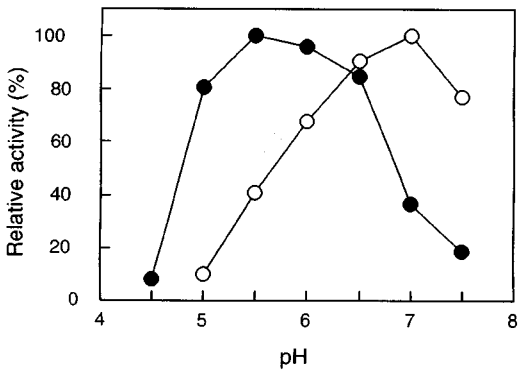


FIG. 2. Effect of pH on the hydrolysis of maltose and the synthesis to TMG. The hydrolysis activities (○) were determined as described in the Experimental Procedures section, except that the pH was varied. The reaction mixture containing 100 μL of 4% maltose, 300 μL of 0.1 M buffer, and 40 μL of α-glucosidase was incubated at 37°C for 20 min. The transglycosylation activities (●) were determined by the measurement of the TMG formed as described in the Experimental Procedures section. The reaction mixture containing 1.0 mL of 40% maltose in 50 mM potassium buffer, 200 μL of 5% TM in dimethyl sulfoxide, and 50 μL of α-glucosidase (5 U) was incubated at 40°C for 20 h. See Scheme 1 for abbreviations.

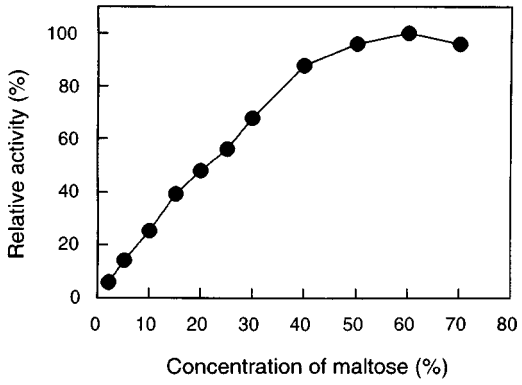


FIG. 3. Effect of maltose concentration on the synthesis of TMG. The reaction mixture containing 1.0 mL of maltose solution, 200 μL of 5% TM in dimethyl sulfoxide, and 50 μL of α-glucosidase (5 U) was incubated at 40°C for 20 h. Relative activity was expressed as a percentage vs. the maximum values obtained. See Scheme 1 for abbreviations.

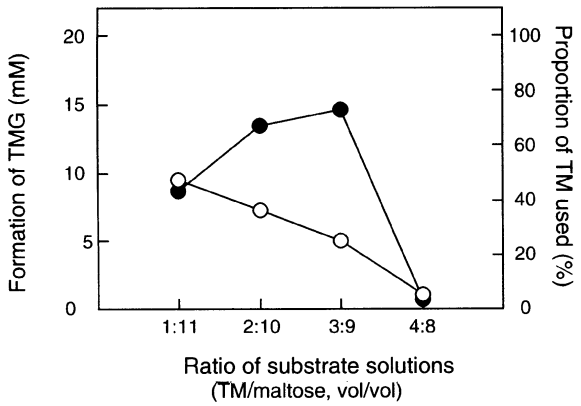


FIG. 4. Effect of TM concentration on the synthesis of TMG. The reaction mixture consisted of 1.2 mL of substrate solution and 50 μL of α-glucosidase (5 U). The ratio between two substrate solutions (5% TM in dimethyl sulfoxide and 40% maltose in 50 mM potassium phosphate buffer, pH 6.0) was varied from 1:11 to 4:8 by volume as indicated. The reaction was carried out at 40°C for 20 h. The amounts of TM and TMG in the reaction mixture were determined by HPLC. The proportion of TM used was expressed as a percentage vs. the initial amounts of TM in the reaction mixture. ○, the TM used; and ●, TMG. See Scheme 1 and Figure 1 for abbreviations.

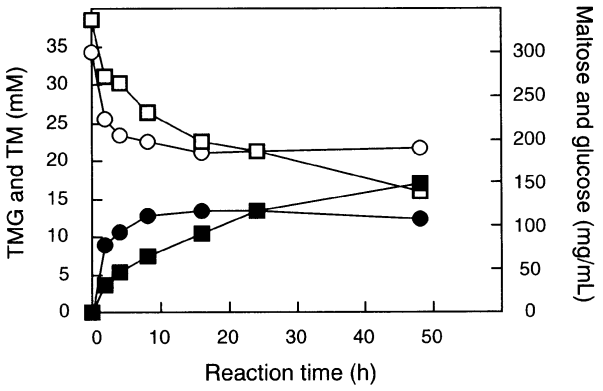


FIG. 5. Time course for the synthesis of TMG. The reaction mixture containing 1.0 mL of 40% maltose solution, 200 μL of 5% TM in dimethyl sulfoxide, and 50 μL of α-glucosidase (5 U) was incubated at 40°C. The amounts of TM, TMG, maltose, and glucose were determined by HPLC as described in the Experimental Procedures section. ●, TMG; ○, TM; □, maltose; and ■, glucose. See Scheme 1 and Figure 1 for abbreviations.

action mixture decreased with the increase of the TM solution ratio. Figure 5 shows the time course for the synthesis of TMG. TM was rapidly converted into TMG with decrease of maltose, and the transglycosylation reaction attained equilibrium after the 16-h incubation. There was no further increase in the amount of TMG even if another fresh α-glucosidase (5 U) was added to the reaction mixture after the 24-h incubation (data not shown).

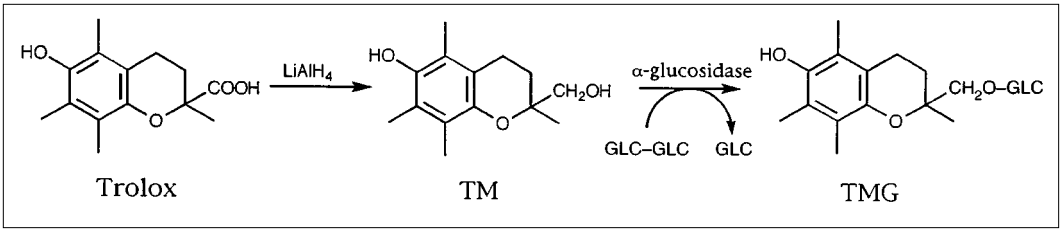
Water solubility of TMG. The solubility (mg/mL) of TMG and other vitamin E analogs in water are TMG, $>1 \times 10^3$; TM, 1.01; Trolox, 0.16. TMG showed the extremely highest solubility ($>1 \times 10^3$ mg/mL) against water among the vitamin E analogs. The value was about 6×10^3 times higher than that of Trolox and was about 1 x 10^3 times than that of TM, respectively.

DPPH scavenging activity. The free-radical scavenging activities of TMG and some other antioxidants were determined by the use of a stable free-radical, DPPH. Comparing the scavenging activities to that of cysteine, 1 molecule of TMG reacted with two molecules of DPPH (27). Thus, one molecule of TMG would trap two molecules of free radicals during the scavenging reactions. This level of activity was nearly the same as those of α-tocopherol, Trolox, and ascorbic acid: antioxidant (20 μM), relative, respectively, are cysteine (1.0); TMG (1.9); TM (1.9); Trolox (2.0); α-tocopherol (1.8); ascorbic acid (1.8).

DISCUSSION

Vitamin E is regarded as the major lipid-soluble antioxidant preventing oxidative attack of membrane lipids and other membrane compounds. The chromanol structure of vitamin E is responsible for the radical scavenging activity and, in particular, the hydroxyl group at the C-6 position is essential for this activity (3,12). If the chromanoxyl ring might be water-soluble, it would scavenge the free radicals produced in aqueous phase efficiently. Therefore, we have studied the synthesis of a water-soluble vitamin E analog: a chromanol compound having hydroxymethyl group, TM, was prepared by the reduction of Trolox; using this derivative, a selectively glycosylation to the hydroxylmethyl group was attempted by α-glucosidase. We have succeeded in the synthesis of a novel water-soluble vitamin E analog, TMG, by this procedure (Scheme 2).

Enzymatic transglycosylation has been utilized to improve the chemical properties of some compounds (18–22). In general, the acceptor (TM) and donor (maltose) should be in a soluble state in the reaction mixture to increase the efficiency of transglycosylation, and the higher the ratio of the acceptor to the donor is necessary. Because TM is barely soluble in water, the addition of a water-miscible solvent to the reaction mixture is required to dissolve the TM in the solution of maltose. The rate of transglycosylation from maltose to TM was examined in the presence of water-miscible organic solvents. When DMSO was used as the water-miscible solvent, the transglycosylation product was found to be formed by α-glucosidase from *Saccharomyces* sp. However, the addition of other solvents, such as ethanol, methanol, acetonitrile, acetone, and *N,N*-dimethylformamide, in the reaction mixture scarcely formed the transglycosylation product (data not shown). Thus, we concluded that DMSO was the most suitable solvent for the synthesis of TMG. On the other hand, a higher concentration of DMSO added in the reaction mixture decreased the efficiency of TMG formation (Fig. 4). In general, enzymes become unstable and lose their activity in a mixture of water and a water-miscible organic solvent. Therefore, the concentration of DMSO should be lowered to minimize the inactivation of α-glucosidase during the glycosylation of TM. The transglycosylation reaction reached to be a plateau at the 16 h incubation under the present conditions (Fig.



SCHEME 2

5). This may be due to the achievement of an equilibrium state, because the addition of another α-glucosidase did not enhance the production of TMG. Furthermore, the concentration of TMG decreased slightly with increase of TM after the 48-h incubation due to the hydrolysis of TMG. There is no other transglycosyl product during the reaction.

The water-solubility of TMG is very high compared with those of another vitamin E analogs, Trolox and TM. Moreover, TMG exhibited a scavenging activity against a stable free-radical, DPPH, as the same degree as those of α-tocopherol, Trolox, and ascorbic acid. α-Tocopherol has been demonstrated to have multiple effects on various diseases. Thus, TMG may be therapeutically a useful antioxidant. We are undertaking further studies to elucidate its antioxidant activity and protective effect against the free-radical damages in biological tissues and fluids.

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Biotransformations of Tocopherols by *Streptomyces catenulae*

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ABSTRACT: *Streptomyces catenulae* catalyzed the oxidation of α-tocopherol to α-tocopherolquinone. Nitrotocopherols isolated from *S. catenulae* grown in defined culture medium containing δ- and γ-tocopherols are formed by a combination of enzymatic nitrate reduction to nitrite, and subsequent nonenzymatic acid-catalyzed nitration. The incorporation of $^{15}N^{18}O_3^-$ into nitrated tocopherols confirmed the origin of the nitrating species. Structures of chromatographically purified products obtained from *S. catenulae* transformations of tocopherols were deduced by spectral (mass spectrometry, ^{1}H-, and ^{13}C-nuclear magnetic resonance) analyses.
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The tocopherols are naturally occurring phenolic benzopyrans which display antioxidant activities *in vivo* and *in vitro*. Since their initial discovery, both chemical and biological systems have been exploited to elucidate their mechanisms of action and to identify the products of oxidation. Much interest has been focused on chemical oxidations of tocopherols catalyzed by agents such as $FeCl_3$ (1), $AgNO_3$ (2), and alkylperoxyl radicals (3–6). Surprisingly no work has been reported on microbial oxidations or transformations of tocopherols. In this paper we describe the results obtained from microbial transformations of α-, δ- and γ-tocopherols.

MATERIALS AND METHODS

Chemicals. α–, δ-, and γ-Tocopherols and α-tocopherolquinone were obtained from Eastman Kodak Company (Rochester, NY). Tocored was prepared as a standard by the oxidation of α-tocopherol with $AgNO_3$ (2). Labeled products including 98% $Na^{15}NO_3$ and 95–98% $H_2{}^{18}O$ were purchased from Cambridge Isotope Laboratories (Andover, MA). Doubly labeled $Na^{15}N^{18}O_3$ was prepared by the exchange reaction between 125 mg $Na^{15}NO_3$ and 0.5 mL $H_2{}^{18}O$ in presence of 0.1 mL of fuming nitric acid in a sealed ampoule at 80°C for 4 d as described in literature (7). The ampoule was opened and the salt was dried by standing at 80°C for two more days. The isotopic composition of labeled nitrate was determined by negative ion fast atom bombardment (FAB) mass spectrometry to show that the $Na^{15}N^{18}O_3$ contained 82% ^{15}N and 81% ^{18}O.

Instrumentation. Thin-layer chromatography (TLC) was carried out on 0.25-mm layers of silica gel GF_{254} (Merck, Darmstadt, Germany) prepared on glass plates with a Quickfit Industries (London) spreader. Plates were air-dried and activated at 120°C for 1 h before use. Mass spectra were obtained by FAB on a Fisons ZAB-HF mass spectrometer (Beverly, MA). ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were obtained on a Bruker WM-360 spectrometer (Bruker Instruments, Billerica, MA) equipped with an IBM Aspect-2000 processor. The signals were assigned on the basis of heteronuclear multiple quantum correlation spectroscopy and heteronuclear multiple bond correlation spectroscopy spectra recorded on a Bruker AMX-600 spectrometer. Tetramethylsilane was used as an internal standard. Chemical shift values are reported in parts per million and coupling constants are given in Hertz. Abbreviations for NMR are as follows: *s*, singlet; *d*, doublet; *t*, triplet; *dd* doublet of doublets; *m*, multiplet. Ultraviolet (UV) spectra were recorded on a Shimadzu (Tokyo, Japan) (UV160U) UV-visible recording spectrophotometer, infrared (IR) spectra on a Nicolet 205 FT-IR spectrometer (Nicolet Instruments, Madison, WI).

Microorganisms. All microorganisms were from the University of Iowa, College of Pharmacy culture collection (UI). They were maintained on Sabouraud-maltose agar slants and stored at 4°C. Microorganisms initially screened for their ability to transform tocopherols were from the following genera: six *Aspergillus* spp., four *Bacillus* spp., three *Candida* spp., one *Corynebacterium* sp., four *Cunninghamella* spp., one *Cylindrocarpon* sp., three *Mucor* spp., two *Mycobacterium* spp., four *Nocardia* spp., two *Penicillium* spp., six *Pseudomonas* spp., three *Rhizopus* spp., one *Rhodotorula* sp., two *Saccharomyces* spp., and five *Streptomyces* spp. *Streptomyces catenulae* (UI-5965) was used for further experiments in biotransformation of tocopherols because of its reproducibly excellent yield of products and relatively fast conversion rates. This microorganism is a substrain of *SS. catenulae* (ATCC 23893) obtained as a result of numerous serial transfers. While the parent strain ATCC 23893 forms uniform white sporulated colonies, the substrain UI-5965 grows as moist asporogenous grey colonies with traces of white spores

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Abbreviations: FT–IR, Fourier transform–infrared; MS, mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

on the colony extremities. Fatty acid analysis for both of these strains using the MIDI/Hewlett-Packard microbial identification system by Analytical Services, Inc. (Williston, VT) confirmed the relationship of both *S. catenulae* strains.

Screening procedure. Cultures were grown in 25 mL of soybean meal–glucose medium (by a two-stage incubation procedure) or in mineral salts–glucose medium (by a one-stage incubation procedure), held in stainless-steel-capped 125 mL DeLong flasks. The soybean meal–glucose medium contained (in g/L) 20 dextrose, 5 yeast extract, 5 soybean meal, 5 NaCl, and 5 K_2HPO_4 in distilled water and was adjusted to pH 7.0 with 6 N HCl. The mineral salts–glucose medium contained in g/L: 2 dextrose, 15 $NaNO_3$, 1.1 KCl, 1.1 NaCl, 0.01 $Ca(NO_3)_2 \cdot 4\ H_2O$, 3.4 $KH_2PO_4 \cdot H_2O$, 4.4 K_2HPO_4, 0.5 $MgSO_4 \cdot 7\ H_2O$, 3×10^{-4} $FeSO_4 \cdot 7\ H_2O$ and 5 mL of a stock solution containing in g/L, 0.29 $ZnSO_4 \cdot 7\ H_2O$, 0.24 $CaCl_2 \cdot 4\ H_2O$, 0.24 $CuCl_2 \cdot 6\ H_2O$, 0.25 $CuSO_4 \cdot 5\ H_2O$, 0.17 $MnSO_4 \cdot H_2O$, in distilled water resulting in a pH of 6.5. Media were autoclaved at 121°C for 15 min before being inoculated. Cultures were incubated with shaking at 250 rpm at 28°C on New Brunswick Scientific G25 Gyrotory shakers. The two-stage incubation procedure consisted of growing cultures in stage I for 60 h and transfering a 10% inoculum of these cultures to 25 mL of medium in 125 mL flasks for the stage II culture. For either incubation protocol, 25 mg of tocopherols in 0.1 mL of *N,N*-dimethylformamide was added to 24-h old cultures which were incubated as before. Samples (4 mL) were taken at 24, 72, and 144 h after addition of substrate. After determination of pH, they were acidified to pH 2.0 with 6 N HCl and extracted with 1 mL of ethyl acetate/propanol (9:1, vol/vol). The organic layers were used for analysis in which 30 to 40 µL samples were spotted on TLC plates which were developed with hexane/isopropanol (97:3 vol/vol). Developed chomatograms were visualized under 254 nm UV light and sprayed with Emmerie-Engel reagent (8) which consisted of a 1:1 mixture of solution A (0.2% $FeCl_3$ in 95% EtOH) and solution B (0.5% α,α′-dipyridyl in 95% EtOH).

Preparative biotransformations of tocopherols. The two-stage incubation process was the same as for screening experiments.

For γ-tocopherol (**1c**), a total of 680 mg of substrate was distributed evenly among 27, 125 mL DeLong flasks each holding 25 mL of 24 h old mineral salts–glucose medium culture. Substrate-containing cultures were incubated for 144 h at which time the reaction media were combined, acidified to pH 2.0, and extracted with ethyl acetate. The organic extract was examined by TLC (hexane–isopropanol 97:3 vol/vol) to show three major spots with R_f 0.69 (yellow), 0.1 (red), and 0.06 (colorless) detected by 254 nm UV light. None of these spots gave purple colors with the Emmerie Engel spray reagent. The extract was dried over Na_2SO_4 and concentrated under vacuum. A brown oily residue was obtained (670 mg).

Biotransformation products obtained from γ-tocopherol (**1c**) were isolated by chomatography over a column of 45 g Baker-40 µm silica gel (hexane/isopropanol 99:1–95:5, vol/vol) to afford two fractions. A pure oily yellow product, **2b** (R_f = 0.69) was obtained in 40% yield (295 mg) and subjected to spectral analysis: UV (hexane) λ_{max} 261, 305 and 415 nm; IR (neat) (ν, cm^{-1}), 2952, 2927, 2868, 1535 (NO_2), 1452, 1411, 1379, 1321, 1261, 1178, 1099; ^{1}H-NMR ($CDCl_3$) δ, 0.84, 0.86, 0.863, 0.864 (4 × *d*, 12 H, *J* = 6.6, 4 × CH_3), 1.27 (*s*, 3H, CH_3-2), 1-1.41 (*m*, 18 H), 1.48-1.61 (*m*, 3 H), 1.69-1.8 (*m*, 2 H, H_2-3), 2.18 (*s*, 3 H, CH_3-8), 2.22 (*s*, 3 H, CH_3-7), 3.01 (*m*, 2 H, H_2-4), 10.73 (*s*, 1 H, OH); ^{13}C-NMR ($CDCl_3$) δ, 12.01 (CH_3-7), 13.27 (CH_3-8), 19.62, 19.72, 20.91, 21.72 (4), 22.6, 22.7, 23.56 (CH_3-2), 24.43, 24.79, 27.96, 30.9 (3), 32.63, 32.78, 37.27, 37.31, 37.37, 37.42, 39.36, 39.58, 75.43 (2), 113.4 (10), 125.3 (7), 131.9 (5), 137.0 (8), 145.2 (9), 148.4 (6); MS (3-NBA) *m/z* 462 (35, $(M + H)^+$), 461 (100, M^+), 446 (34), 445 (25), 444 (44), 431 (27), 430 (29), 428 (27), 416 (21), 236 (60), 234 (41), 230 (21), 220 (98), 204 (88).

The second fraction contained a mixture of red spots at R_f = 0.1 and R_f 0.05, **2b**, starting material (R_f = 0.3), and traces of compounds sensistive to Emmerie-Engel reagent (Rf = 0.7 and 0.73). Those at R_f 0.7 and 0.73 were also detected in controls containing no cultures, and thus were not analyzed further. Repeated chomatography attempts to isolate the red spot at R_f 0.1 failed because of its apparent instability on silica gel. It was indirectly identified as tocored (**3**) by comparison with a standard. Its degradation product at R_f 0.05 was isolated and identified as tocored dimer **4** which had the following physical properties: UV (hexane) λ_{max} 208, 288, 440 nm; IR (neat) (ν, cm^{-1}) 2952, 2927, 2867, 1677, 1648, 1622, 1582, 1462, 1393, 1326, 1189, 910, 733; ^{1}H-NMR ($CDCl_3$) δ, 0.83-0.87 (*m*, 24 H, 8 × CH_3), 1.0-1.45 (*m*, 38 H), 1.45-1.85 (*m*, 14 H), 2.05 (*s*, 6 H, 2 × CH_3-7), 2.43 (*m*, 4 H, 2 × H_2-4), 2.64 (*s*, 4 H, 2 × H_2-8); ^{13}C-NMR ($CDCl_3$) (2 carbons for each peak) δ, 11.77, 15.46, 19.57, 19.71, 21.26, 22.6, 22.69, 24.4, 24.45, 24.76, 26.36, 27.94, 29.18, 32.68, 32.75, 37.24, 37.27, 37.31, 37.4, 39.33, 39.72, 82.32, 111.2, 14.9, 145.2, 162.3, 177.6, 180.8; MS (thioglycerol) *m/z* 860 (11), 859 (12, $(M + H)^+$), 445 (11), 432 (38), 431 (100), 329 (11), 205 (170), 167 (96).

For δ-tocopherol (**1b**), a total of 545 mg of substrate was added at 24 h to *S. catenulae* cultures to a final concentration of 1 mg/mL of culture medium. Substrate-containing cultures were harvested at 144 h following substrate addition, the cultures were combined, adjusted to pH 2.0 with 6 N HCl, and exhaustively extracted with ethyl acetate. The oily extract obtained was examined by TLC (hexane/isopropanol 97:3) to show a yellow product at R_f 0.65 representing about 10% yield.

Product from δ-tocopherol. δ-Tocopherol (**1b**) afforded a metabolite at R_f 0.65 in 10% yield as estimated by TLC. Column chomatography over 40 g of silica gel with hexane/isopropanol 99:1–95:5 (vol/vol) gave 55 mg pure metabolite identified spectrally as **2a**: UV (hexane) λ_{max} 259, 296, and 408 nm; IR (CCl_4) (ν, cm^{-1}) 2955, 2930, 1548 (NO_2), 1460, 1320, 1258, 1220, 1005, 980; ^{1}H NMR ($CDCl_3$) δ, 0.84, 0.86, 0.864, 0.865 (4 × *d*, 12H, *J* = 6.6, 4 × CH_3), 1.27 (*s*, 3H, CH_3-2), 1-1.65 (*m*, 21H), 1.76 (*m*, 2H, H_2-3), 2.20 (*s*, 3H, CH_3-8), 3.05 (*m*, 2 H, H_2-4), 6.82 (*s*, 1 H, H-7), 10.32 (*s*, 1 H, OH);

^{13}C-NMR ($CDCl_3$) δ, 17.25 (8a), 19.61, 19.72, 20.86, 21.89 (4), 22.6, 22.69, 23.57 (2a), 24.42, 24.78, 27.96, 30.71 (3), 32.64, 32.77, 37.26, 37.35, 37.37, 37. 41, 39.35, 39.52, 75.53 (2), 116.3 (10), 118.4 (7), 132.1 (5), 138.8 (8), 145.9 (9), 149.6 (6); MS (3-NBA) *m/z* 448 (18, $(M + H)^+$), 447 (53, M^+), 432 (26), 430 (30), 414 (19), 402 (16), 222 (53), 206 (100), 191 (28), 190 (64), 189 (52), 188 (40), 182 (20), 176 (28), 175 (27), 166 (44).

For α-tocopherol, 530 mg of substrate was evenly distributed among 21, 125 mL DeLong flasks containing 25 mL of 24-h old *S. catenulae* cultures grown in mineral salts medium. Incubations were continued for 144 h when cultures were combined, acidified to pH 2 with 6 N HCl, and exhaustively extracted with ethyl acetate. TLC (hexane–isopropanol 97:3) evaluation of the organic extract revealed two bands detected by 254 nm UV light. These were light yellow (R_f = 0.18) and red (R_f = 0.14) and neither of these was sensitive to Emmerie-Engel reagent.

Product from α-tocopherol. Chromatography of the extract obtained from *S. catenulae* UI 5965 over 40 g silica gel (hexane–isopropanol 99:1 to 95:5, vol/vol) failed to resolve the yellow biotransformation product at R_f 0.18 from the red one at R_f 0.1. However it accomplished the removal of traces of other contaminating products to afford a 460 mg mixture of the yellow and red products. The second column using the same system afforded two fractions for spectral analysis. The yellow oily fraction (R_f 0.18, 195 mg) was identified as α-tocopherol quinone (**5**) by comparison with a standard: ^{1}H NMR ($CDCl_3$) δ 0.84-0.88 (*m*, 12 H, 4 × CH_3), 1-1.65 (*m*, 26 H), 2.0 (*s*, 6 H, 2 × CH_3), 2.04 (*s*, 3 H, CH_3), 2.55 (*m*, 2 H, CH_2); ^{13}C-NMR ($CDCl_3$) δ 11.85, 12.18, 12.26, 19.62, 19.66, 21.25, 21.34, 22.53, 22.63, 24.4, 24.7, 26.46, 27.87, 32.68, 32.7, 37.19, 37.35, 37.55, 39.27, 40.11, 42.2, 72.48, 140.1, 140.3, 140.4, 144.4, 187.1, 187.5.

The second fraction was again a mixture of yellow and red compounds. The red compound was identified as tocored by chromatographic comparison with standard.

Nitration of γ–tocopherol with $Na^{15}NO_3$ and $Na^{15}N^{18}O_3$. Biotransformations were conducted as previously described with 7 mg tocopherol in 5 mL medium containing 35 mg of unlabeled $NaNO_3$, 35 mg $Na^{15}NO_3$ or 35 mg doubly labeled $Na^{15}N^{18}O_3$ in 50 mL DeLong flasks. To avoid possible label exchanges, nitrate was added after sterilization of the medium. After 144 h incubation, the mixtures were separately acidified and extracted with ethyl acetate. The 5-nitro-γ-tocopherol product of each reaction was isolated by short column chomatography on Baker-40 μm silica gel (hexane/isopropanol 97:3 vol/vol) and analyzed by mass spectrometry.

Conversion of nitrate to nitrite by S. catenulae *(UI-5965).* Cultures of *S. catenulae* (UI-5965) were grown as usual in 25 mL of mineral salts–glucose medium. Samples taken at intervals of 0, 4, 24, 48, 72, 96, 120, and 168 h were diluted 20-fold with distilled water. For analysis, 1 mL of diluted sample was mixed with 0.5 mL Griess reagent (9) (1:1 vol/vol mixture of 1% sulfanilamide in water and 0.1% naphthylethylenediamine in 2% phosphoric acid). Mixtures were vortexed and allowed to stand at room temperature for 15 min before their absorbances were measured at 543 nm. Nitrite concentrations were estimated by comparisons of samples with a standard curve over a range of 0.1 to 1.5 mmol/L prepared freshly with sodium nitrite in water.

RESULTS

A total of 117 microorganisms were screened for their abilities to catalyze biotransformations of tocopherols. Initially, all cultures were grown in a soybean meal–glucose medium using a standard two-stage incubation protocol. No bioconversions were detected. A series of *Streptomyces* spp. were then grown in mineral salts–glucose medium, and apparent tocopherol biotransformations were reproducibly observed with *S. viridosporus* (T-7A), *S. badius* (UI-252), *S. griseus* var. *erizensis* (NRRL-3242), and *S. catenulae* (UI-5965).

Of all organisms screened for their abilities to transform tocopherols, a substrain of *S. catenulae* derived by multiple serial transfer of the culture reproducibly achieved conversions of tocopherols in good yield. Colonies of the substrain (UI 5965) and the parent *S. catenulae* American Type Culture Collection (Rockville, MD) (ATCC) 23893 grown on Sabouraud maltose agar were similar in size, but different in sporulation. When viewed from the bottoms of Petri plates, both colonies were dark grey in color. However, the original ATCC culture produced abundant white spores while the substrain was asporogenous except for traces of white spores on colony extremities. The MIDI/Hewlett-Packard microbial identification was used to demonstrate that both *S. catenulae* strains produced the same fatty acid profile, thus, confirming their relationship to one another. This substrain was used for preparative-scale incubations with α-, δ-, and γ-tocopherols as substrates.

When the biotransformation of **1c** to **2b** was conducted in medium containing 98% $Na^{15}NO_3$, ^{15}N-nitro-γ-tocopherol was obtained (Fig. 1). The mass spectrum of **2b** gave a molecular ion at *m/z* 462 (Fig. 1B) whereas that for unlabeled **2b** occurs at *m/z* 461 (Fig. 1A). From incubations with $Na^{15}N^{18}O_3$, mass spectral analysis demonstrates that **2b** was labeled in both the nitrogen (about 80%, *m/z* 462), and oxygen atoms. From relative intensities of peaks at M + 2 (*m/z* 464) and M + 4 (*m/z* 466) (Fig. 1C), oxygen enrichment in **2b** was estimated to be mono-^{18}O ($^{15}N^{18}OO_2$, 12%) and di-oxygen labeled ($^{15}N^{18}O_2O$, 2%).

DISCUSSION

Microbial transformations have been studied with practically every naturally occurring and biologically active compound. Surprisingly, there have been no reports in the literature dealing with microbial conversions of tocopherols. Initial screening with 117 cultures using our standard biotransformation protocol in a soybean meal–glucose medium failed to identify any microorganisms capable of yielding metabolites with α-, δ-, or γ-tocopherols as substrates. Upon changing to a sim-

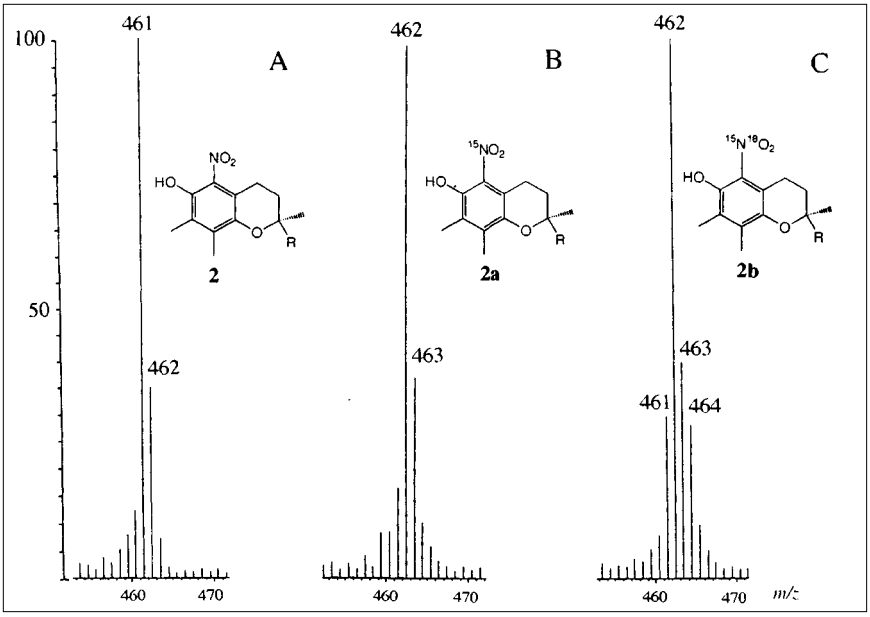
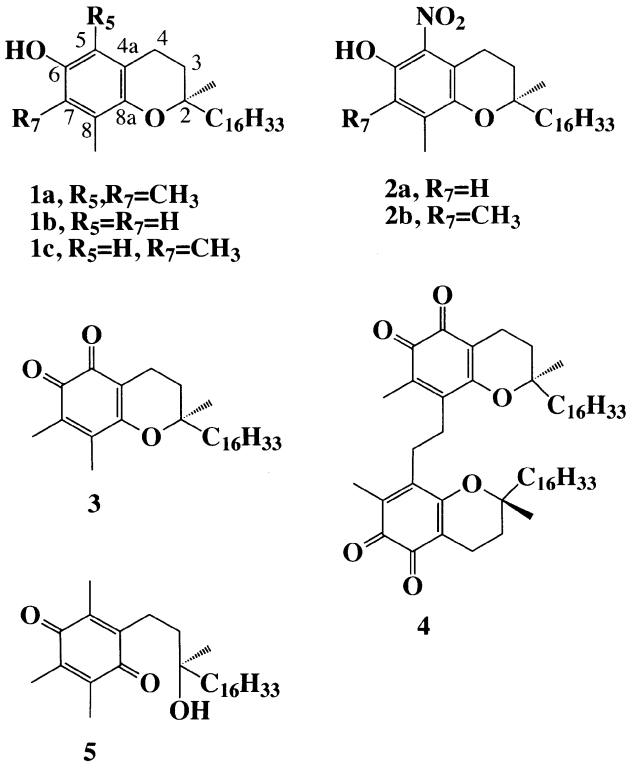


FIG. 1. Molecular ions from mass spectra of 5-nitro-γ-tocopherols produced by biotransformation of γ-tocopherol (**1c**) in the presence of A) NO_3^-, B) $^{15}NO_3^-$, and C) $^{15}N^{18}O_3^-$.

pler mineral salts culture medium, several Streptomycetes showed the capacity for producing metabolites of these tocopherols. Metabolites of yellow and red colors were obtained, but only traces of those demonstrating positive colors with the Emmerie-Engel reagent were obtained. The most reproducible and major yields of metabolites were obtained with a substrain of *S. catenulae*, and this culture was used to preparatively form metabolites from the three tocopherols.

The major metabolite obtained in 40% yield from γ-tocopherol (**1c**) was identified as **2b** by UV, MS, ^{1}H-, and ^{13}C-NMR spectroscopy (Scheme 1). The 5-nitro-γ-tocopherol obtained with *S. catenulae* was identical to **2b** described in the literature (10). This product known as tocoyellow (10) was obtained in studies concerned with the oxidation of γ-tocopherol with rat insulinoma cells and with nitrite. An additional unstable product obtained from *S. catenulae* cultures was tentatively identified as tocored (**3**) by comparison with a standard of the orthoquinone prepared by chemical oxidation (2). The unstable tocored (**3**) dimerizes during chomatography to **4**, tocored-dimer, which was isolated and characterized. With α-tocopherol as substrate, the only identified product was **5**, or α-tocopherolquinone obtained by oxidation of the tocopherol ring system. With δ-tocopherol (**1b**), the only identifiable metabolite was 5-nitro-δ-tocopherol (**2a**) which was characterized spectrally. Nitrations of tocopherols occurred in excellent yield. Since microbiological nitrations of substrates is a relatively rare reaction, we investigated the nature of this reaction further.

Microbial nitration has been previously reported for compounds with tocopherol related structures. An *o*-nitrophenol metabolite was isolated from a mixed culture of soil microorganisms grown in presence of a 6-chromanol derivative (11). Also, 5-chromanol derivatives related to canabinoids were nitrated by cultures of *Pseudomonas putida* (12). However, as-



SCHEME 1

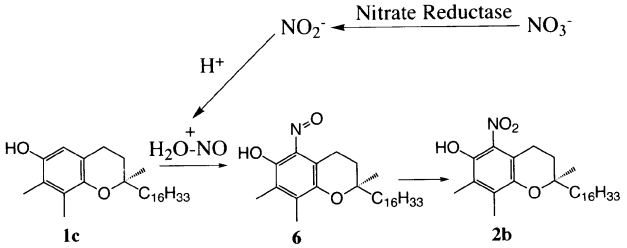
pects of the mechanism of microbial nitration of these phenolic substrates were not determined.

Besides their antioxidant activities, tocopherols are potent antinitrosating agents (13). α- and γ-Tocopherols prevent the formation of nitrosamines by very rapidly reacting with the nitrosating species. Kamm *et al.* (14) demonstrated that *in vitro* α- and γ-tocopherols accelerate the disappearance of nitrite in simulated gastric fluids. Formation of α-tocopherolquinone was observed during the reaction of α-tocopherol while the product from the reaction of γ-tocopherol was not identified. However, nitration of γ-tocopherol to **2b** occurred in hexane solutions containing nitrogen dioxide (NO_2) (10). Interestingly, **2b** was also identified in RINm5F cells containing γ-tocopherol. The source of nitrating species was presumed to be from the endogenous formation of nitric oxide (NO·) formed in these mammalian cells (10). These previous studies suggested the possibility that Streptomycetes produced nitrated tocopherols by converting inorganic nitrate in the culture medium to nitrating species. To test this possibility, a series of experiments were conducted using labeled nitrate.

These results indicate that bacterial transformation of nitrate to an active nitrating species had occurred. A reasonable pathway for nitrate reduction to active nitrating species would involve initial reduction of NO_3^- to NO_2^-, which under acidic conditions can form protonated nitrous acid (H_2ONO^+) as a phenol nitrosating or nitrating species. Nitrosation of the phenol in the first step to **6** and subsequent oxidation of **6** to **2b** (Scheme 2) would account for the loss of label in the nitrated product **2b**. Low recovery of enrichment in ^{18}O in the product could thus be explained by two phenomena: an exchange reaction between the nitrate and water could occur during the enzymatic reaction, and chemical nitration by nitrite in acidic medium.

The reduction of nitrate to nitrite is a common reaction among denitrifing bacteria. However, the reduction of NO_3^- to NO_2^- by *Streptomyces* species has been rarely reported. The ability of *S. catenulae* to produce nitrite during growth in the mineral salts medium was evaluated using the Griess reaction. Nitrite was detected after 24 h of incubation and reached levels of 8 to 14 mM after 48 h.

Since nitrite levels of 8–14 mM were detected in growing cultures of *S. catenulae,* we examined the possible reactivity of tocopherols toward nitrite. γ-Tocopherol was previously reported to react very rapidly with sodium nitrite under faintly acidic conditions yielding **2b** (15). The pH of the medium was nearly neutral thoughout *S. catenulae* incubation. However, during work-up of the reaction mixture, samples were acidified to pH 2 before extraction for analysis or isolation. To determine whether the nitration of γ-tocopherol to **2b** occurred biocatalytically or by chemical catalysis following bacterial reduction of nitrate to nitrite, cultures were grown in mineral salts–glucose medium containing nitrite in place of nitrate. Incubations and controls containing γ-tocopherol but no microorganism were subjected to both acidic and neutral extractions. The nitrated product **2b** was detected only after acidic extraction in both inoculated and control flasks. This is consistent with nitration which occurred by microbial reduction



SCHEME 2

of nitrate to nitrite followed by chemical reaction. None of the identified products was formed in control incubations and only in mineral salts medium restricted to Streptomyces.

Many bacteria are capable of catalyzing *N*-nitrosation reactions at neutral or almost neutral pH (16–18), the most effective being those having nitrate reductase and nitrite reductase activities. It would be interesting to verify the antinitrosation effect of γ-tocopherol with these bacteria and to identify the metabolites for better understanding of the mechanism of action of tocopherols.

This work describes the first microbial transformation study of tocopherols. *Streptomyces catenulae* (UI-5965) nitrate reductase activity was demonstrated by the measurement of NO_2^- in culture media, and by the conversion of $^{15}N^{18}OO$-labeled nitrate into labeled 5-nitro-γ-tocopherol. The results demonstrate that nitration or nitrosation reactions are possible with *Streptomycetes* when they can enzymatically reduce nitrate to nitrite in acidic soils.

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METHOD

Tandem Mass Spectrometric Approach for Determining Structure of Molecular Species of Aminophospholipids

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ABSTRACT: Aminophospholipids, including glycerophosphatidylethanolamine, glycerophosphatidylserine and their Lyso analogous, have been analyzed by positive and negative ion liquid secondary ion ionization coupled to tandem mass spectrometry. The mass spectra of aminophospholipids obtained by tandem mass spectrometers with different configuration (liquid secondary ion-electric-magnetic sector coupled to quadrupole mass analyzer (low-energy collision) or electric-magnetic sector (high-energy collision), as well as electrospray ionization-quadrupole mass analyzer combined with quadrupole mass analyzer (low-energy collision), are compared. The mass spectra produced by low-energy collisionally induced dissociation of the deprotonated molecules from aminophospholipids contain fragment ions for characterizing polar head moieties as well as fatty acid composition and position. The mass spectra generated by high-energy collisionally induced dissociation of both protonated and deprotonated molecules from aminophospholipids show numerous product ions for identifying polar heads, composition, and location of fatty acid chains in molecular species. Triple quadrupole mass spectrometer with electrospray ionization exhibits remarkable superiority in detection sensitivity. Liquid secondary ion with electric-magnetic sector coupled to quadrupole mass analyzer or electric-magnetic sector instrument has the advantage of the capability of properly determining location of fatty acid chains in molecular species. This paper also describes an approach for structurally analyzing aminophospholipid species as 9-fluorenylmethyloxycarbonyl derivatives by positive and negative ion liquid secondary ion mass spectrometry and high-energy collisionally induced dissociation tandem mass spectrometry. It has been found that the derivatives of glycerophosphatidylethanolamine and glycerophosphatidylserine can readily be analyzed by the negative ion liquid secondary ion and tandem mass spectrometric methods.

Lipids 32, 85–100 (1997).

Aminophospholipids, including glycerophosphatidylethanolamine (GPE), glycerophosphatidylserine (GPS), lysophosphatidylethanolamine (LysoPE) and lysophosphatidylserine (LysoPS), are present as essential components in biological membranes and located preferentially on the inner monolayer. The structural diversity of these lipid molecules is mainly due to (i) different polar head moieties, such as phosphoethanolamine and phosphoserine, linked to phosphate at the *sn*-3 position of the glycerol backbone; (ii) a variety of diacyl-, alkyl-acyl, and alkenyl-acyl-linked fatty chains esterified at the *sn*-1 and *sn*-2 positions, and (iii) location of the double bond(s) (between 1 and 6) within unsaturated fatty chains with a number of carbon atoms (between 14 and 22). Lyso analogous of aminophospholipids, which usually contain only one fatty chain (acyl or alkyl or ether group) at the *sn*-1 position of the glycerol backbone, are found as minor components in membranes. A number of studies have demonstrated that the phospholipids have played an important role in the fields of pharmacology (1–4), immunology (5), and oncology (6).

Several approaches have been taken in the chemical analysis of aminophospholipids: thin-layer chromatography (7,8), and normal-phase high-performance liquid chromatography (HPLC) (9,10) are commonly employed for the separation and identification of phospholipid classes; gas chromatography is an effective method for analyzing fatty acid composition within aminophospholipids rather than for determining structures of individual molecular species. The identification of molecular species and of fatty acid chain location by means of reversed-phase HPLC separation of chromatography-purified aminophospholipid class, following by enzymatic treatment and gas chromatographic analysis of hydrolyzed fatty acids (11,12) is laborious, time-consuming, and material-consuming. The approach is suitable for the routine analysis of aminophospholipids, but is inappropriate in structurally elucidating molecular species from new natural sources.

Mass spectrometry has been employed for the structural analysis of molecular species of glycerophospholipids since 1969 (13), and some papers have reported studies of aminophospholipids using a variety of ionization modes, including electron impact (13), chemical ionization (14), field desorption (15), liquid secondary ion (LSI) [or fast atom bombardment (FAB) (16)], thermospray (17), electrospray ionization (ESI)(18) and matrix-assisted laser desorption (19). This method allows to analyze molecular species of aminophospholipids from picomole or lower quantities of material, and the structure of a single species can be tentatively designated

Abbreviations: CID, collisionally induced dissociation; ESI, electrospray ionization; FAB, fast atom bombardment; Fmoc, 9-fluorenylmethyloxycarbonyl; GPE, glycerophosphatidylethanolamine; GPS, glycerophosphatidylserine; HPLC, high-performance liquid chromatography; LSI, liquid secondary ion; LysoPE, lysophosphatidylethanolamine; LysoPS, lysophosphatidylserine.

by conventional mass spectrometry. Classical methods coupled to ESI mass spectrometry, such as HPLC combined with ESI, is appropriate in initially identifying the glycosylated derivatives of aminophospholipid molecular species (20). However, when applied in characterizing an aminophospholipid molecule from molecular species mixtures or crude extracts, the structural information on the composition and location of fatty acid chains in the glycerol backbone in conventional mass spectra is usually insufficient. For example, identical ions at *m/z* 792 were observed in conventional positive ion LSI mass spectra of GPE classes from both rainbow trout liver and water snail *Lymnaea stagnalis* kidney, corresponding to the protonated molecules of GPE 40:6 [*X*:*Y* (for example GPE 40:6), where X is the total carbon number of fatty acids esterified at the *sn*-1 and *sn*-2 position and *Y* is the total number of the degree of unsaturation of fatty acid chains. The code GPE *X*:*Y* or GPS *X*:*Y* is usually used for expressing general structural information on polar heads and fatty acids in molecular species, which is obtained by conventional mass spectrometry.]. For further details, GPE 18:0–22:6 [*N*:*M*-*n*:*m* (for example GPE 18:0–22:6), where *N* is the total number of carbon in the *sn*-1 fatty chain, *M* is the total number of double bond(s) in the fatty acid esterified at the *sn*-1 position; *n* is the total number of carbon in the *sn*-2 fatty chain, *m* is the total number of double bond(s) in the fatty acid esterified at the *sn*-2 position. The code GPE *N*:*M*-*n*:*m* or GPS *N*:*M*-*n*:*m* is often used for indicating the nature of polar head moieties and the positions of two fatty chains in the glycerol backbone, which can be obtained by tandem mass spectrometry.] is present in the former and GPE 20:2–20:4 being in the latter, and the correct structure of a molecular species can readily be designated based on information obtained by CID tandem mass spectrometry (21,22).

Tandem mass spectrometry (23), by means of mass selection of ions and followed by CID mass spectrometric analysis in multisector or multiquadrupole instruments, is an increasingly important method for characterizing the structure of a wide range of phospholipids. Although field desorption, thermospray, and matrix-assisted laser desorption ionization can yield protonated and deprotonated molecules of molecular species of aminophospholipids, the irreproducibility of $[M - H]^-$ or $[M + H]^+$ ions, generated by the ionization modes above, makes it difficult to carry out CID experiments. The tandem mass spectrometric approach of $[M - H]^-$ ions from aminophospholipids, produced by negative ion LSI (FAB) and ESI ionization, has been widely applied to the structural identification of molecular species (24,22). The major advantage of this method is that it is able to readily provide diagnostic information on the characterization of polar head moieties and the composition and location of fatty acid chains in lipid molecules. Jensen and Gross (25) as well as Murphy and Harrison (26) have given excellent reviews on the analysis of intact glycerophospholipids by negative ion LSI (FAB) mass spectrometry and CID tandem mass spectrometry. Positive ion LSI (FAB) mass spectrometry have been reported to yield abundant protonated molecules of aminophospholipids (27,28), and product ions obtained by CID of $[M + H]^+$ ions of aminophospholipid species are structurally informative (29). Mechanisms of high energy (keV, main electronic excitation) and low energy (eV, mainly vibrational excitation) CID of ions have been well discussed (30).

On the other hand, the qualitative determination of aminophospholipids is also of importance in investigating biological and pharmacological functions of these lipid species. Because trace amounts of aminophospholipids are present in some biological samples, such as plasma lipoproteins, HPLC coupled to chromophore detection is difficult to assay both underivatized aminophospholipid class and molecular species. The main reason is that aminophospholipid molecules lack a common moiety that can be readily detected under ultraviolet or fluorescence detection. The facile route chosen in the structural modification of aminophospholipids is the replacement of ethanolamine or serine phosphate by a colorimetric group or the introduction of a chromophore group to polar head moieties of the species. The direct introduction of various groups [a biphenyl carbonyl (31) or a dimethylaminonaphthalene-5-sulfonyl (32) or a naphthyl (33) or a trinitrophenyl (34) moiety] to aminophospholipids followed by HPLC separation of the derivatives has been achieved, but the methods overlooked the structural elucidation of derivatized molecular species. The introduction of 9-fluorenylmethyloxycarbonyl (Fmoc) group into the primary amine in peptides has been well documented (35,36) because of advantages of (i) the formation of stable derivatives and (ii) very low detecting levels under fluorescence detection. By using this mode, Fmoc group has been readily introduced into the primary amine in polar head moieties of GPE, GPS, and their lyso compounds with a good yield (more than 92%). A satisfactory separation of Fmoc derivatives of aminophospholipid classes and molecular species has been achieved on aminopropyl normal-phase and octadecylsilyl reversed-phase HPLC column (not shown). The low detecting levels under fluorescence detection is as little as 125 femtomoles. Details on the analysis of aminophospholipids as Fmoc derivatives by HPLC will be reported in a separate paper.

Work described in the present study was involved first of all in comparing (i) CID mass spectra of molecular species of underivatized aminophospholipids, obtained by positive and negative ion LSI and ESI coupled to low-energy CID (the results of ESI low-energy CID of aminophospholipids were collected from Refs. 37–39 cited in the paper) as well as positive and negative ion LSI combined with high-energy CID experiments; and then (ii) positive and negative ion LSI mass spectrometry and tandem mass spectrometry of Fmoc derivatives of molecular species of GPE and GPS. The advantage and disadvantage of the three sort of tandem mass spectrometers [the hybrid (EBQQ) instrument, the triple quadrupole (QQQ) mass spectrometer and the four-sector (EBEB) machine], which are commercially available and are being used in biomedical mass spectrometry laboratories, in structurally analyzing molecular species of aminophospholipids are discussed.

EXPERIMENTAL PROCEDURES

Chemicals. All aminophospholipid species used were purchased from Sigma Company (St. Louis, MO). All natural aminophospholipids used were prepared by the methods as described (40). Diethanolamine (DEA) obtained from Fluka (Buchs, Switzerland) was used as liquid matrix in this study.

Mass spectrometry. Positive and negative ion LSI high-energy CID tandem mass spectrometric experiments were performed with a CONCEPT II HH four-sector (EBEB) mass spectrometer (Kratos Analytical Ltd., Manchester, United Kingdom), equipped with LSI source and a scanning-array detection system as reported (41). Positive and negative ion LSI low-energy CID spectra of $[M + H]^+$ and $[M - H]^-$ ions of aminophospholipid species were obtained on a VG 70SEQ (EBQQ) instrument (Fisons Analytical, Manchester, United Kingdom). Cesium ions with a translational energy of 18 keV were applied as the ion beam. The accelerating voltage was 8 kV. Precursor ions were mass-selected with a resolution of 1,000 using the electric-magnetic sector (EB) part of the mass spectrometer. CID was carried out in quadruple collision cell with argon as collision gas at a pressure of approximately 5×10^{-6} bar and at a collision energy of 50 eV. Product ion spectra were obtained in the continuous accusation mode with accumulation of 15–20 scans at unit resolution of the quadrupole mass analyzer. The surface precipitation technique of LSI (FAB) mass spectrometry has proven to be particularly useful in analyzing all kinds of phospholipids (42), especially serine containing phospholipids (43,44).

RESULTS

Comparison of High- and Low-Energy CID of Molecular Species of Aminophospholipids

Glycerophosphatidylethanolamine

Low-energy CID. The fragmentation observed in low-energy CID spectra of diacyl- and alkenylacyl-GPE molecular species is summarized in Table 1. Data of ESI CID of

TABLE 1
Fragmentation of GPE Generated by CID Tandem Mass Spectrometry[a]

				Polar head group	Fatty acid composition and location
1,2-Diacyl-GPE	LSI/ESI	$[M + H]^+$	L-CID	$[M + H$ – phosphoethanolamine$]^+$	
			H-CID	$[M + H$ – phosphoethanolamine$]^+$ [phosphoethanolamine + H]$^+$ [ethanolamine]$^+$	$[R_2COOCHCH_2]^+$(a)[b], $[R_1COOCHCH_2]^+$(w)[c] $[R_2COOCHCHOH]^+$, $[R_1COOCHCHOH]^+$ $[R_2COOCHCHCOOCH_2CH_2]^+$, $[R_1COOCHCHCOOCH_2CH_2]^+$
	LSI/ESI	$[M - H]^-$	L-CID		$[R_1COO]^-$(w), $[R_2COO]^-$(a), $[M - H - R_2CHCO]^-$
			H-CID		$[R_1COO]^-$(w), $[R_2COO]^-$(a)
	ESI	$[M + Na]^+$	L-CID	$[M - Na$-phosphoethanolamine$]^+$ $[M + Na - CH{=}CHNH_2]^+$ $[M + Na - 123]^+$	
1,2-Diacyl-dimethyl-GPE	LSI	$[M + H]^+$	L-CID	$[M + H$ – dimethylphospho-ethanolamine$]^+$	
			H-CID	$[M + H$ – phosphodimethyl-ethanolamine$]^+$ [phosphodimethylethanolamine]$^+$ [diethylethanolamine]$^+$	
Alkenylacyl- or alkylacyl-GPE	LSI	$[M + H]^+$	L-CID	$[M + H$ – phosphoethanolamine$]^+$	
			H-CID	$[M + H$ – phosphoethanolamine$]^+$ [phosphoethanolamine + H]$^+$ [ethanolamine]$^+$	$[R_1COOCHCH_2]^+$, $[R_1COOCHCHOH]^+$, $[R_1COOCHCHCOOCH_2CH_2]^+$
	LSI/ESI	$[M - H]^-$	L-CID		$[R_2COO]^-$
			H-CID		$[R_2COO]^-$
	ESI	$[M + Na]^+$	L-CID	$[M + Na$ – phosphoethanolamine$]^+$ $[M + Na - CH{=}CHNH_2]^+$ $[M + Na - 123]^+$	

[a]Low-energy CID (L-CID) and high-energy CID (H-CID) tandem mass spectrometry; GPE, glycerophosphatidylethanolamine; CID, collisionally induced dissociation; LSI, liquid secondary ion; ESI, electrospray ionization. [b](a) Abundant ion. [c](w) Weak ion.

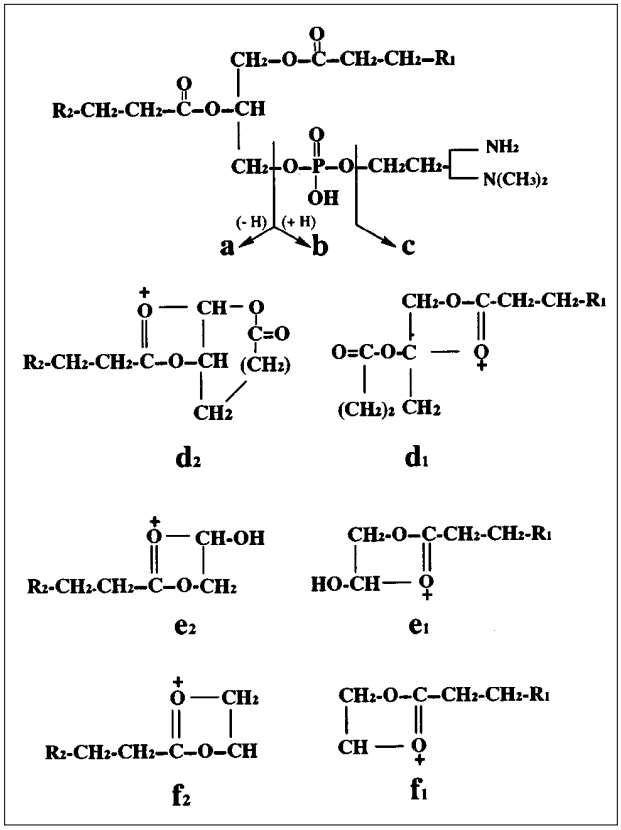
GPE was collected from References 37 and 38 cited in this paper.

Positive ion CID mass spectra. CID of LSI-generated $[M + H]^+$ ion (*m/z* 692) of GPE 16:0-16:0 yields a single fragment at *m/z* 551 (not shown), formed by the loss of phosphoethanolamine from its $[M + H]^+$ ion. The abundant information of this ion may be used for characterizing the polar-head group of GPE. None of other informative signals corresponding to fatty acid chains can be observed in the spectrum. The identical ion pattern was also found in the CID spectrum of *N,N*-dimethyl-GPE 16:0-16:0 species (not shown). CID experiments of LSI-generated $[M + H]^+$ ions of alkenylacyl-GPE species gave identical results as those of diacyl-GPE species (see Table 1). CID spectra (37) of ESI-generated $[M + H]^+$ ions of diacyl- and alkenylacyl-GPE species are similar to those of the related species obtained by the LSI CID experiment. CID of ESI-generated $[M + Na]^+$ ions of diacyl- and alkenylacyl-GPE mainly produces $[M + Na - 141]^+$ and $[M + Na - 163]^+$ ion (38), which are useful for characterizing the polar head of GPE (Table 1).

Negative ion CID spectra. Carboxylate anions resulting from CID of LSI-generated $[M - H]^-$ ions of GPE are produced in an abundant ratio of approximately 1:3 for fatty acyl chains esterified at the *sn*-1 and *sn*-2 positions, respectively, and by which the composition and location of two carboxylate chains in a GPE species can be identified properly. Tandem mass spectrometry of the $[M - H]^-$ ion from GPE 40:7 from rainbow trout liver (not shown) gives quite abundant carboxylate anions at *m/z* 281 (18:1; oleic acid) and *m/z* 327 (22:6; docosahexaenoic acid), relating to the two fatty acyl chains. The intensity of the signal at *m/z* 327, resulting from the chain at the *sn*-2 position, is more abundant than that of the anion at *m/z* 281, corresponding to the fatty acyl chain esterified at the *sn*-1 position (45). A weak peak, due to $[M - R_2CO]^-$, appeared in the spectrum as well (not shown). This species is identified as GPE 18:1-22:6. CID spectrum of LSI-generated $[M - H]^-$ ion of alkenylacyl-GPE species (45) contains only one carboxylate anion corresponding to $[R_2COO]^-$ ion. Alkenylacyl-GPE species can be distinguished from alkylacyl-GPE species by the combination of mild acid hydrolysis of the former with subsequent tandem mass spectrometric analysis of the latter (45). These ion patterns have been confirmed by CID of ESI-generated $[M - H]^-$ ion of diacyl- and alkenylacyl-GPE (37,38) (Table 1).

High-energy CID. Product ions in high-energy CID mass spectra of diacyl- and alkenylacyl-GPE species are listed in Table 1.

Positive ion CID mass spectra. CID of LSI-generated $[M + H]^+$ ions of GPE species induces several fragmentation processes (Scheme 1 shows proposed structures of product ions yielded by CID of $[M + H]^+$ ions of GPE, based on results from Ref. 29 cited in this paper). Product ions, $[M + H - \text{ethanolamine phosphate}]^+$, $[\text{phosphoethanolamine} + H]^+$ and $[CH_2CH_2NH_2]^+$, due to **a**, **b**, and **c** fragments, are useful for characterizing the nature of polar head moieties of different GPE. Figure 1 shows CID spectra of LSI-generated $[M + H]^+$ ions of GPE 16:0-16:0 (Fig. 1A) and *N,N*-dimethyl-GPE 16:0-16:0 (Fig. 1B) species. Peaks at *m/z* 551 (**a** type fragmentation) are formed by the loss of phosphoethanolamine or *N,N*-dimethylphosphoethanolamine from their protonated molecules at *m/z* 692 (the former) and *m/z* 720 (the latter), respectively. Alternative fragment ions for identifying polar head groups of the two GPE also appear at *m/z* 141 and 169 ($[\text{phosphoethanolamine} + H]^+$ and $[\text{*N,N*-dimethylphosphoethanolamine} + H]^+$), due to **b** type fragmentation, as well as at *m/z* 44 and 72 ($[\text{ethanolamine}]^+$ and $[\text{*N,N*-dimethylethanolamine}]^+$), due to **c** type fragmentation. **d**, **e**, and **f** type product ions (Scheme 1) are useful in identifying fatty acid composition in GPE species. The location of fatty acyl chains is indicated by the presence of $[R_2COOCHCH_2]^+$ and $[R_1COOCHCH_2]^+$ (f_2 and f_1 ions) as well as $[R_2COOCHCH_2CH_2O]^+$ and $[R_1COOCHCH_2CH_2O]^+$ ions (e_2 and e_1 ions) and by the intensity differences of f_2 and f_1 fragments, respectively. The abundance of $[R_2COOCHCH_2]^+$ ion is greater than that of $[R_1COOCHCH_2]^+$ peak, and the ion intensity ratio of f_2/f_1 is always more than 1. This conclusion was based on results obtained by LSI-generated $[M + H]^+$ ions of various asymmetric GPE species. Figure 2A exhibits CID spectrum of LSI-generated $[M + H]^+$ ion of trout liver GPE 40:6. This species is identified as GPE 18:0-22:6 since the abundance of the ion resulting from the *sn*-2 position (*m/z* 354 due to f_2 ion) is richer than that of the peak relating to the



SCHEME 1

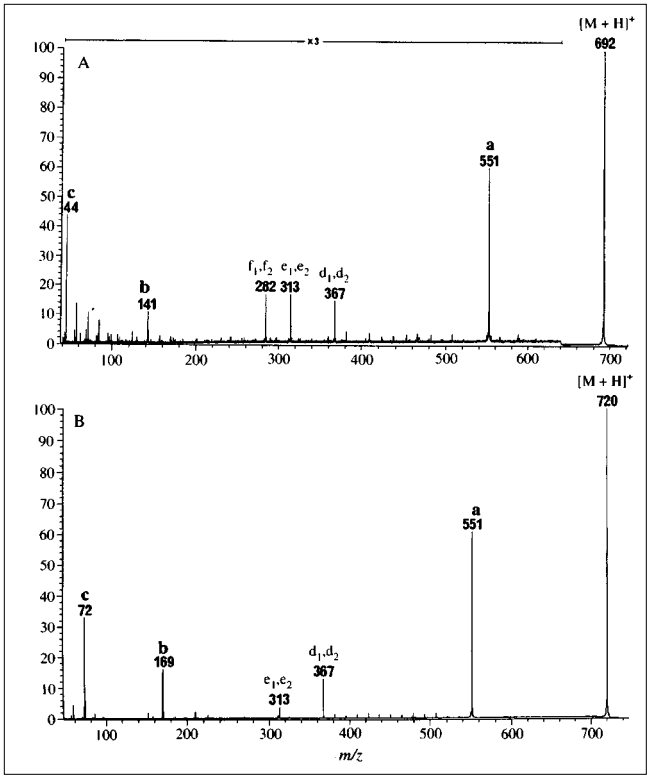


FIG. 1. High-energy CID spectra of protonated molecules of GPE 16:0-16:0 (A) and *N,N*-dimethyl-GPE 16:0-16:0 (B).

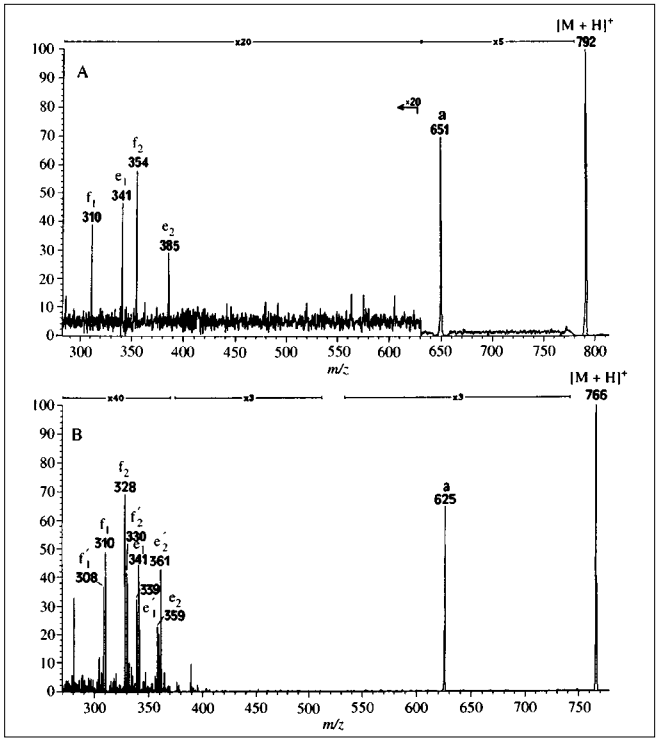


FIG. 2. High-energy CID spectra of protonated molecules of rainbow trout liver GPE 18:0-22:6 (A) and *Lymnaea stagnalis* kidney GPE 18:0-20:5 (B) and GPE′ 18:1-20:4 (B).

sn-1 position (*m/z* 310 due to f_1 ion) (29). **d** and **e** type fragments (*m/z* 365 and 361 due to e_2 and e_1 ions) cannot be differentiated unambiguously for diagnostics of the fatty acyl chain position, and the former is absent in CID spectra of $[M + H]^+$ ions of molecular species containing polyunsaturated fatty acids at the *sn*-2 position. The identical fragment pattern is also observed in CID mass spectrum of LSI-generated $[M + H]^+$ ions of GPE 18:0-20:5 and GPE 18:1-20:4 from *L. stagnalis* kidney (Fig. 2B). CID of $[M + H]^+$ ion of *N,N*-dimethyl-GPE 16:0-16:0 (Fig. 1B) yields product ions due to **d** and **e** cleavages, which are useful for identifying the composition of fatty acyl chains in the species (Table 1). This approach also provides the possibility of analyzing GPC species by CID of $[M + H]^+$ ions of *N,N*-dimethyl-GPE species derived from GPC molecules after chemical treatment (46). The study is in progress and will be reported in a separate paper. Diacyl-GPE can be distinguished from alkenylacyl-GPE species depending upon the absence of $[R_1COOCHCH_2]^+$ and $[R_1COOCHCH_2CH_2O]^+$ ions in the CID spectrum (29).

Negative ion CID spectra. High-energy CID mass spectra of $[M - H]^-$ ions of GPE species contain identical information as those obtained by low-energy CID of $[M - H]^-$ ions (Table 1). The CID mass spectrum of LSI-generated $[M - H]^-$ ion of GPE 16:0-18:2 (not shown) exhibits fragment ions due to the fatty acyl chains, and the intensity of the carboxylate anion lost from the *sn*-2 position (*m/z* 279; $[R_2COO]^-$) is higher than that of the ion resulting from the *sn*-1 position (*m/z* 255; $[R_1COO]^-$). The molecular species of plasmalogen GPE can be identified by absence of $[R_1COO]^-$ ion in CID spectra (45).

Lysophosphatidylethanolamine

Low-energy CID. Product ions yielded by low-energy CID of monoacyl- and alkenyl-LysoPE (plasmalogen) species are listed in Table 2. Data of ESI CID is collected from Reference 39 cited in the paper.

Positive ion CID spectra. CID of LSI-generated $[M + H]^+$ ion (*m/z* 482) of LysoPE 18:0 induces two fragments at *m/z* 341 and 464, corresponding to [M + H − ethanolamine phosphate]$^+$ and $[M + H - H_2O]^+$ (shown in Fig. 3A), for characterizing the polar head group and fatty acid chain in the species. CID of LSI-generated $[M + H]^+$ ion (*m/z* 466) of plasmalogen LysoPE 18:0 (Fig. 3B) produces three product ions at *m/z* 295 ([M + H − ethanolamine phosphate − $CH_2OH]^+$, *m/z* 312 ([M + H − ethanolamine phosphate − $CH_2]^+$, and *m/z* 448 ([M + H − $H_2O]^+$). [M + H − ethanolamine phosphate]$^+$ ion is absent in CID spectrum of plasmalogen LysoPE. CID of ESI-generated $[M + Na]^+$ ions (*m/z* 502) of LysoPE 18:1 regioisomers produces abundant fragment ions at *m/z* 459 ([M + Na − 43]$^+$), *m/z* 441 ([M + Na − 61]$^+$), *m/z* 339 ([M + Na − 163]$^+$) and *m/z* 164 ([phosphoethanolamine + Na]$^+$. The structures of the two regioisomers can be distinguished by the intensity differences of the product ions (38) derived from CID of their $[M + Na]^+$ ions (Table 2).

Negative ion CID spectra. CID of LSI-generated $[M - H]^-$

TABLE 2
Fragmentation of LysoPE Generated by CID Tandem Mass Spectrometry[a]

				Polar head group	Fatty acid composition and location
1-Acyl-LysoPE	LSI	$[M + H]^+$	L-CID	$[M + H -$ phosphoethanolamine$]^+$ $[M + H - H_2O]^+$	
			H-CID	$[M + H -$ phosphoethanolamine$]^+$ $[M + H - H_2O]^+$, $[(CH_2)_2NH_2]^+$	$[R_1COOHCH_2CH]^+$
		$[M - H]^-$	L-CID		$[R_1COO]^-$
			H-CID		$[R_1COO]^-$
Alkenyl-LysoPE	LSI	$[M + H]^+$	L-CID	$[M + H - CH_2$-phosphoethanolamine$]^+$, $[M + H - H_2O]^+$	$[R_1CH{=}CHOCH_2CH]^+$
			H-CID	$[(CH_2)_2NH_2]^+$	$[R_1CH{=}CHOCH_2CH]^+$
1-Acyl-LysoPE	ESI	$[M + Na]^+$	L-CID	$[M + Na - 43]^+$, $[M + Na - 61]^+$(a)[b] $[M + Na - 163]^+$(w)[c]	
2-Acyl-LysoPE	ESI	$[M + Na]^+$	L-CID	$[M + Na - 43]^+$, $[M + Na - 61]^+$(w) $[M + Na - 163]^+$(a)	
1-Acyl-LysoPE	ESI	$[M - H]^-$	L-CID	[glycerol phosphoethanolamine $-H_2O]^-$	$[R_1COO]^-$
Alkenyl-LysoPE	ESI	$[M - H]^+$	L-CID	[glycerol phosphoethanolamine $-H_2O]^-$	$[O{=}CHCH(CH_2)nCH_3]^-$

[a]Low-energy CID (L-CID) and high-energy CID (H-CID) tandem mass spectrometry; LysoPE, lysophosphatidylethanolamine. See Table 1 for other abbreviations. [b](a) Abundant ion. [c](w) Weak ion.

ion of LysoPE produces only an abundant $[R_1COO]^-$ anion (not shown) for identifying fatty acyl chain in the species. This anion is absent in CID mass spectra of LSI-generated $[M - H]^-$ ion of plasmalogen LysoPE (not shown). CID of ESI-generated $[M - H]^-$ ions of LysoPE species yields abundant carboxylate anion, corresponding to $[R_1COOH]^-$. Plasmalogen LysoPE can be identified by both the appearance of $[O{=}CHCH(CH_2)_{15}CH_3]^+$ ion and absence of $[R_1COO]^-$ anion in the spectra, compared with CID of $[M - H]^-$ of LysoPE molecules (39) (Table 2).

High-energy CID. The fragmentation observed in high-energy CID spectra of monoacyl- and alkenyl-LysoPE (plasmalogen) species is summarized in Table 2.

Positive ion CID spectra. CID of LSI-generated $[M + H]^+$ ions of LysoPE yields fragment ions of $[M + H -$ ethanolamine phosphate$]^+$ and $[$ethanolamine$]^+$ for determining the polar head group. The fatty acyl chain at the *sn*-1 position can be identified as the peak of $[M + H - PO_4H_2CH_2CH_2NH_2]^+$. Plasmalogen GPE species is detected by the absence of the ion due to $[M + H -$ ethanolamine phosphate$]^+$.

Negative ion CID spectra. Product ions yielded by CID of LSI-generated $[M - H]^-$ ions of LysoPE and plasmalogen LysoPE are identical to those obtained by the low-energy CID tandem mass spectrometry of the related species (see Table 2).

Glycerophosphatidylserine

Low-energy CID. The fragmentation obtained by low-energy CID of GPS species is summarized in Table 3. Data of ESI CID of GPS was collected from References 37 and 38 cited in this paper.

Positive ion CID mass spectra. Abundant protonated molecules of GPS species ($[M + H]^+$) can be produced by LSI mass spectrometry using the surface precipitation technique (28). CID of LSI-generated protonated molecule of GPS 16:0-16:0 (not shown) only yields a single fragment ion at *m/z* 551 ($[M + H -$ phosphoserine$]^+$), formed by the loss of serine phosphate from its $[M + H]^+$ ion. None of product ions due to fatty acid structure appears in the CID spectrum. CID of ESI-generated $[M + Na]^+$ ion of GPS species has been reported (37,38) to yield product ions corresponding to $[M + Na - CHCHCOOH(NH_2)]^+$, $[M + Na -$ phosphoserine$]^+$ and $[$phosphoserine + Na$]^+$ for characterizing the polar head moiety (Table 3).

Negative ion CID spectra. Abundant deprotonated molecule ($[M - H]^-$) of GPS species can be readily produced by LSI mass spectrometry using the surface precipitation technique (28). The polar head moiety of GPS is detected by the ion of $[M - H - CH_2CH(NH_2)COOH]^-$, due to $(M - 88)$. The characterization of fatty acyl chains esterified can be identified by appearances of anions, corresponding to $[M - 88 -$

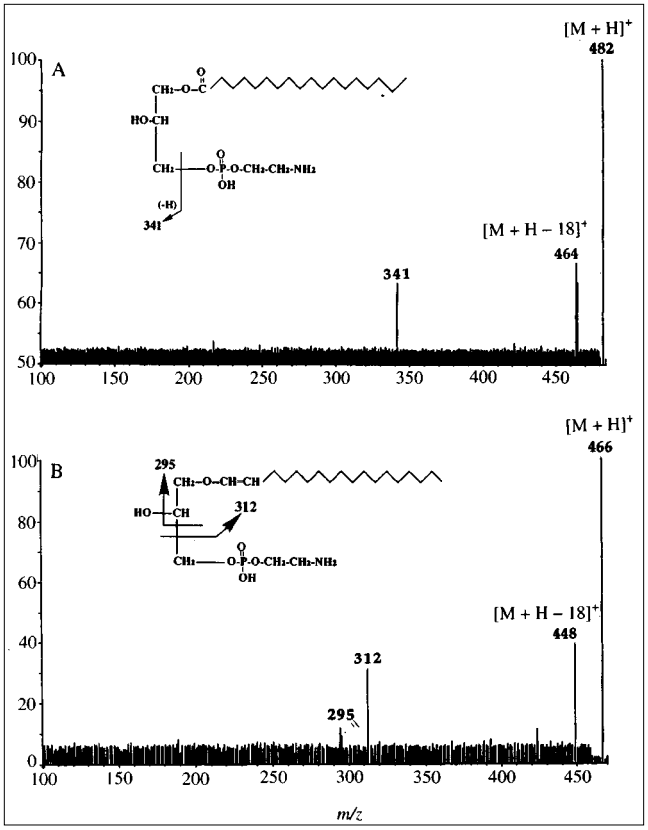


FIG. 3. Low-energy CID spectra of protonated molecules of LysoPE 18:0 (A) and plasmalogen LysoPE 18:1 (B).

$R_2COOH]^-$, $[R_2COO]^-$ and $[R_1COO]^-$, and the intensity of carboxylate fragment derived from the *sn*-1 position ($[R_1COO]^-$) is higher than that of the ion relating to fatty acid esterified at the *sn*-2 position ($[R_2COO]^-$) (not shown). This conclusion was based on results obtained by CID of LSI-generated $[M - H]^-$ ions of GPS 16:0-18:1, GPS 18:0-18:1 and GPS 18:0-22:6. The relative intensity ratio of $[R_1COO]^-/[R_2COO]^-$ is slightly affected by the chainlength of fatty acids and the degree of unsaturation of fatty acyl groups, but is always greater than 1. CID of ESI-generated $[M - H]^-$ ion of GPS species gives the result (38) identical to that obtained by CID of LSI-generated $[M - H]^-$ ion of GPS (Table 3).

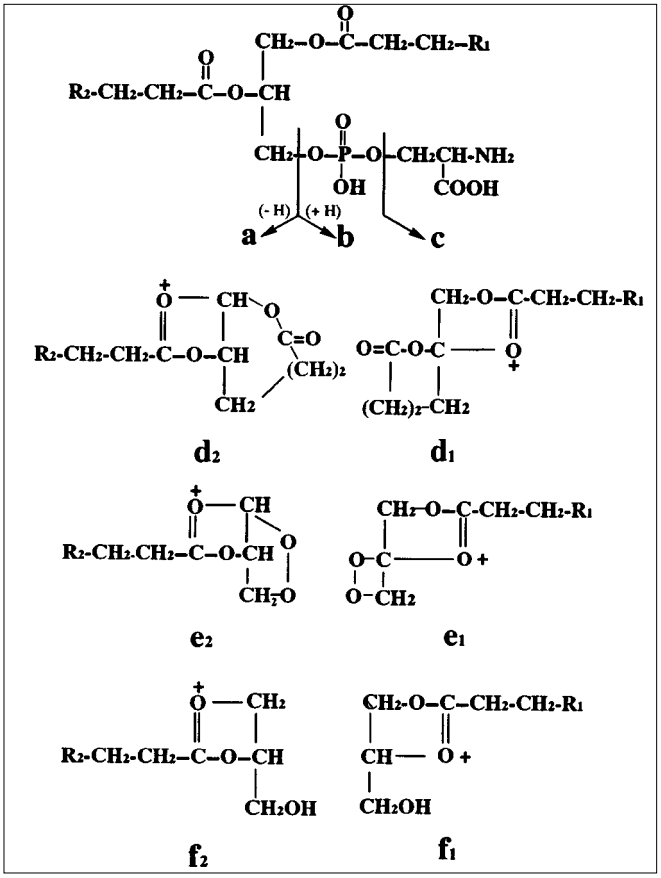
High-energy CID. The fragmentation yielded by high-energy CID of $[M + H]^+$ and $[M - H]^-$ ions of GPS is listed in Table 3.

Positive ion CID spectra. CID of LSI-generated $[M + H]^+$ ions of the molecular species of GPS produces six fragmentation processes (Scheme 2 shows proposed structures of product ions obtained by CID of $[M + H]^+$ ions of GPS, based on results from Reference 29 cited in this paper). The polar head of GPS is characterized by **a**, **b,** and **c** type fragment ions, due to $[M + H -$ phosphoserine$]^+$, $[$phosphoserine $+ H]^+$ and $[CH_2CH(NH_2)COOH]^+$. CID of $[M + H]^+$ ions of GPS 16:0-16:0 (Fig. 4A) and GPS 18:0-18:1 (Fig. 4B) also yield the product ions, due to **d**, **e**, and **f** type fragmentation, for the identification of fatty acid composition in the species. Positions of the two fatty acyl chains in GPS 18:0-18:1 (4B) can

TABLE 3
Fragmentation of GPS Generated by CID Tandem Mass Spectrometry[a]

			Polar head group	Fatty acid composition and location
1,2-Diacyl-GPS (LSI)	$[M + H]^+$	L-CID	$[M + H -$ phosphoserine$]^+$	
		H-CID	$[M + H -$ phosphoserine$]^+$ $[$phosphoserine $+ H]^+$ $[CH_2CH(COOH)NH_2]^+$	$[R_1COOCH(CH_2)_2CH]^+$(a)[b], $[R_2COOHCH(CH_2)_2CH]^+$(w)[c] $[R_1COOCHO(CH_2)_2O]^+$, $[R_2COOCHO(CH_2)_2O]^+$ $[R_1COO(CH)_2CH_2COO(CH_2)_2]^+$, $[R_2COO(CH)_2CH_2COO(CH_2)_2]^+$
	$[M - H]^-$	L-CID	$[M - CH(COOH)NH_2]^-$(M-88)	$[R_1COO]^-$(a), $[R_2COO]^-$(w) $[M - 88 - R_2COOH]^-$(a), $[M - 88 - R_1COOH]^-$(w) $[M - 88 - R_2CO\text{-}H]^-$(a), $[M - 88 - R_1CO\text{-}H]^-$(w)
		H-CID	$[M - CH(COOH)NH_2]^-$(M-88)	$[R_1COO]^-$(a), $\{R_2COO]^-$(w) $[M - 88 - R_2COOH]^-$(a), $[M - 88 - R_1COOH]^-$(w) $[M - 88 - R_2CO\text{-}H]^-$(a), $[M - 88 - R_1CO\text{-}H]$_(w)
1,2-Diacyl-GPS (ESI)	$[M + Na]^+$	L-CID	$[$phosphoserine $+ Na]^+$ $[M + Na -$ phosphoserine$]^+$ $[M + Na - CH_2CH(COOH)NH_2]^+$	
	$[M - H]^-$	L-CID		$[R_1COO]^-$(a), $[R_2COO]^-$(w), $[M - 88 - R_2CO\text{-}H]^-$(a) $[M - 88 - R_1CO\text{-}H]^-$(w), $[M - 88 - R_2COOH]^-$(a), $[M - 88 - R_1COOH]^-$(w)

[a]Low-energy CID (L-CID) and high-energy (H-CID) CID tandem mass spectrometry; GPS, glycerophosphatidylserine. See Table 1 for other abbreviations. [b](a) Abundant ion. [b](w) Weak ion.



SCHEME 2

be identified on the basis of intensity differences of f_2 (*m/z* 339) and f_1 (*m/z* 341) ion (see Scheme 2). The abundance of the f_2 is weaker than that of the f_1 (f_2/f_1 ratio <1). The explanation for obtaining the opposite intensity ratio of product ions of GPS, compared with that of GPE, is that LSI-generated $[M + H]^+$ ions of GPS are probably the fragment ions of $[M + H + \text{diethanolamine}]^+$ peaks, the solvated protonated molecules of GPS. The diversity of kinetic energy of the selected precursor ions makes the different patterns (29).

Negative ion CID spectra. High-energy CID spectra of LSI-generated $[M - H]^-$ ions of GPS species contains structural information, which includes: the characterization of polar head, the mass of fatty acids in species and location of fatty acyl chains. Figure 5 illustrates CID spectra of $[M - H]^-$ ions of underivatized 16:0-18:1 (a) and 18:1-16:0 (b) isomers. Signals at *m/z* 673 are formed by losses of 88 Da ($[CH_2CH(NH_2)COOH]^-$) from the deprotonated molecules of the two species. Product ions at *m/z* 255 (palmitic acid; 16:0) and 281 (oleic acid; 18:1) correspond to fatty acyl chains. However, intensities of a couple of anions give an opposite ratio, compared with those of carboxylate fragment ions obtained by CID of $[M - H]^-$ ions of underivatized GPE species, and the abundance of the peaks eliminated from the *sn*-2 fatty acid residues are weaker than those of the ions derived from the acids at the *sn*-1 position (47,48). This fact is mainly due to the favorable formation of glycerophosphatidic acid fragment ($[M - 88]^-$) that governs the fragmentation derived from it (49). The diversity of the pathways to yield fatty acid anions leads to the differences in the relative abundance of carboxylate product ions derived from $[M - H]^-$ parent ions (47).

Lysophosphatidylserine

Low-energy and high-energy CID. Product ions yielded by low- and high-energy CID of $[M + H]^+$ ion from LysoPS species are listed in Table 4.

Positive ion CID mass spectra. Low-energy CID of LSI-generated $[M + H]^+$ ions of LysoPS species produces fragment ion $[M + H - \text{phosphoserine}]^+$, which is useful for characterizing the polar head moiety and fatty acid chain in the species (not shown) (Table 4). High-energy CID of LSI-generated $[M + H]^+$ ion of LysoPS 14:0, shown in Figure 6, yields ions at *m/z* 299 $[M - PO_3CH_2CH(NH_2)COOH]^+$, *m/z* 285 $[M + H - \text{phosphoserine}]^+$, and *m/z* 88 $[CH_2CH(NH_2)COOH]^+$ for characterizing polar head moiety and fatty acid chain in the species.

Negative ion CID spectra. Low- and high-energy CID of LSI-generated $[M - H]^-$ ion of LysoPS species produces only an abundant anion $[R_1COO]^-$, which relates to the fatty acyl chain in LysoPS species (not shown) (Table 4).

TABLE 4
Fragmentation of LysoPS Generated by CID Tandem Mass Spectrometry[a]

			Polar head group	Fatty acid composition and location
Acyl-LysoPS (LSI)	$[M + H]^+$	L-CID	$[M + H - \text{phosphoserine}]^+$	
		H-CID	$[M + H - \text{phosphoserine}]^+$ $[M + H - \text{phosphoserine} + O]^+$ $[CH_2CH(COOH)NH_2]^+$	
	$[M - H]^-$	L-CID		$[R_1COO]^-$
		H-CID		$[R_1COO]^-$

[a]Low-energy CID (L-CID) and high-energy (H-CID) tandem mass spectrometry; LysoPS, lysophosphatidylserine. See Table 1 for other abbreviation.

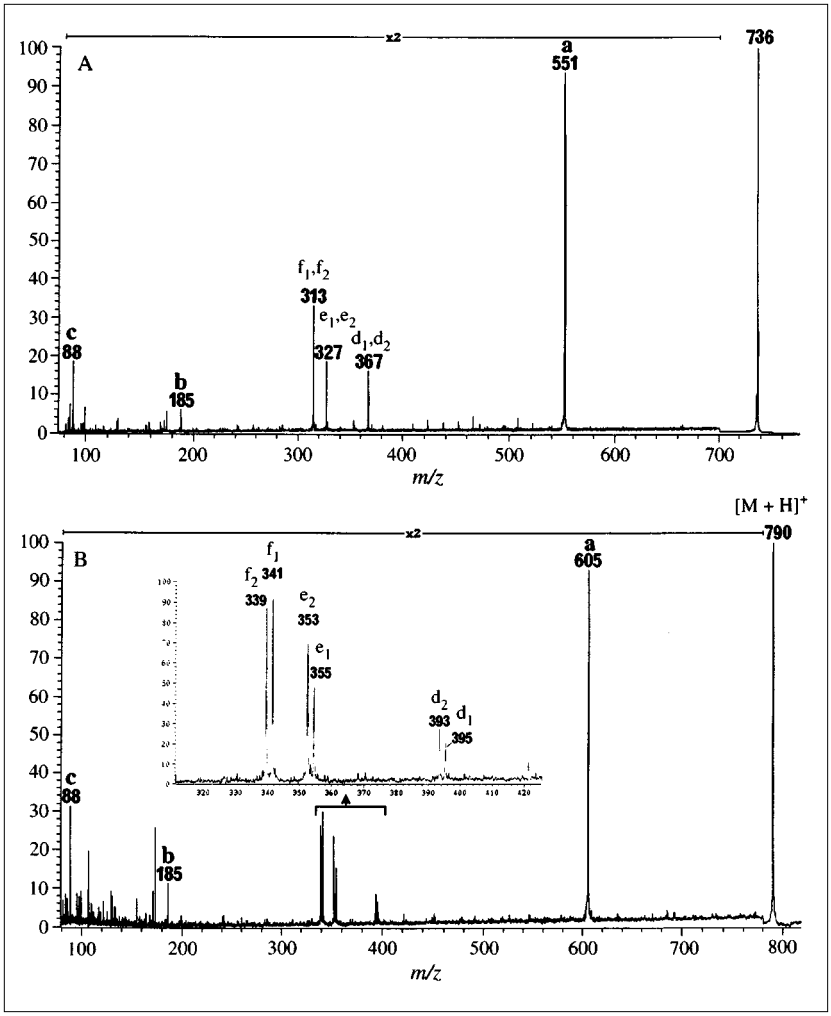


FIG. 4. High-energy CID spectra of protonated molecules of GPS 16:0-16:0 (A) and GPS 18:0-18:1 (B).

Liquid Secondary Ion Mass Spectrometry and Tandem Mass Spectrometric Analysis of Molecular Species of GPE and GPS as Fmoc Derivatives

Fmoc derivatives of GPE. *Positive ion LSI and high-energy CID mass spectra.* The protonated molecules of Fmoc GPE species are absent in conventional LSI mass spectra. CID of $[M + Na]^+$ ion (*m/z* 935) of Fmoc GPE 16:0-16:0 (not shown) yields mainly an abundant ion at *m/z* 386, corresponding to [Fmoc-phosphoethanolamine + $Na]^+$, which is useful for characterizing the polar head group.

Negative ion LSI and high-energy CID mass spectra. Figure 7A shows the conventional negative ion LSI mass spectrum of Fmoc-GPE18:0-20:4. The peak at *m/z* 988 corresponds to the deprotonated molecule of this derivative. The ion at *m/z* 766 is formed by the loss of a Fmoc group from its $[M - H]^-$ parent ion. Fragment ions due to fatty acyl chains are present at *m/z* 283 (stearic acid) and 303 (arachidonic acid). The conventional mass spectrum of reversed-phase HPLC-purified Fmoc derivative of GPE from rat kidney exhibits an abundant peak at *m/z* 960, which relates to the deprotonated molecule of 36:4 species (not shown). The ion at *m/z* 738 ($[M - H - 44]^-$) is formed by the loss of an ethanolamine group from the $[M - H]^-$ ion. However, it is difficult to identify fatty acyl chains in the species, and the structural information regarding acyl chain positions in Fmoc derivatives of 1,2-diacyl-GPE species can only be obtained by CID of LSI-generated $[M - H]^-$ ions. Figure 7B exhibits CID spectrum of LSI-generated $[M - H]^-$ ion (*m/z* 960) of Fmoc-36:4 species. Product ions appeared at *m/z* 255 (palmitic acid) and 303 (arachidonic acid) furnish correct information on the composition and location of two fatty acyl chains. This species is identified as GPE 16:0-20:4 since the abundance of the anion at *m/z* 303 is richer than that of *m/z* 255 peak (47). This determination is in a good agreement with results published (50). Alkenylacyl-GPE (plasmalogen GPE) can be distinguished from diacyl-GPE depending on the absence of an anion, corresponding to the fatty chain at the *sn*-1 position (47). The fragment pattern of high-energy CID spectra of Fmoc GPE species is identical to

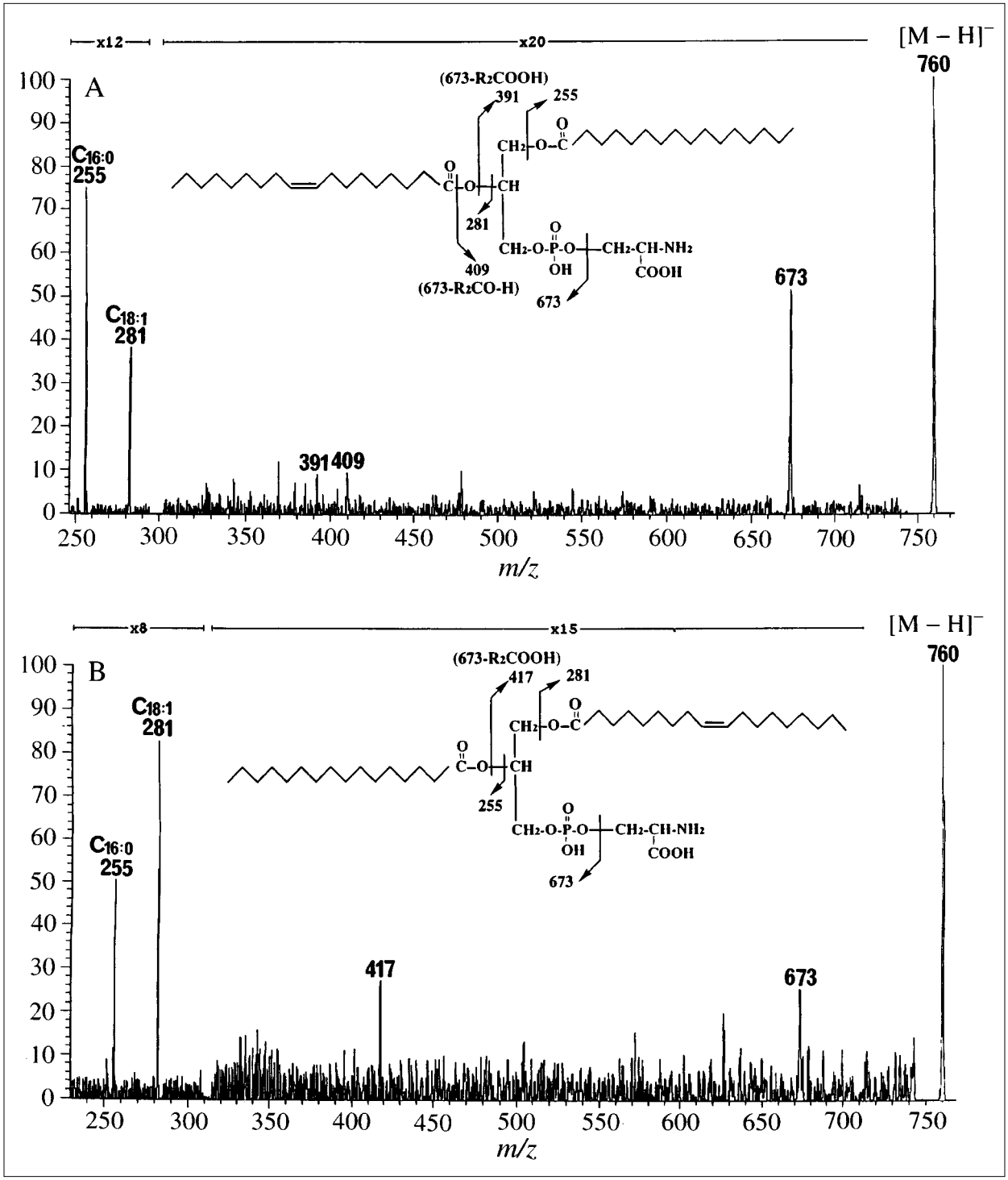


FIG. 5. High-energy CID spectra of deprotonated molecules of GPS 16:0-18:1 (A) and GPS 18:1-16:0 (B).

that of high-energy CID of underivatized GPE molecules (see Table 1).

Fmoc derivatives of GPS. *Positive ion LSI and high-energy CID mass spectra.* Both $[M + H]^+$ and $[M + Na]^+$ ions are absent in the conventional positive ion LSI mass spectra of Fmoc derivatives of GPS species. CID of $[M + 2\ Na - H]^+$ ion (*m/z* 1002) of Fmoc-16:0-16:0 species (not shown) generates product ions mainly at *m/z* 957 ($[M + 2\ Na - H - COOH]^+$), 693 ($[M + 2\ Na - H -$ Fmoc-serine$]^+$), 509 ($[M + 2\ Na - H -$ Fmoc-serine $- C_{13}H_{28}]^+$), 437 ($[M + 2\ Na - H -$ Fmoc-serine $- R_1COOH]^+$ or $[M + 2\ Na - H -$ Fmoc-serine $- R_2COOH]^+$), 143 ($[H_2PO_4 + 2\ Na]^+$), and 125 ($[H_2PO_4 + 2\ Na - H_2O]^+$) (29).

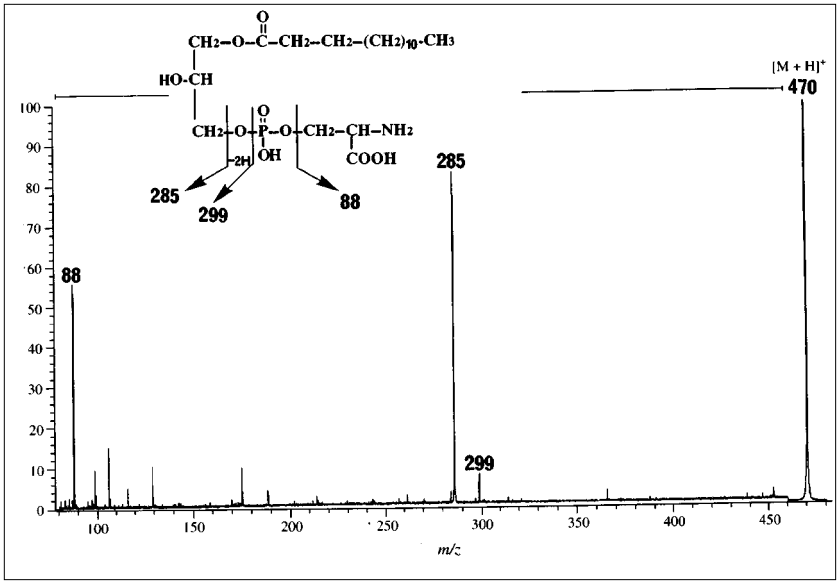


FIG. 6. High-energy CID spectrum of protonated molecule of LysoPS 14:0.

Negative ion LSI and high-energy CID mass spectra. The conventional negative ion LSI mass spectrometry produces abundant deprotonated molecules of Fmoc derivatives of GPS species. Figure 8 exhibits the conventional mass spectrum of Fmoc-16:0-18:1 (A) and Fmoc-18:1-16:0 (B) isomers. Anions at *m/z* 982 correspond to the deprotonated molecules of the two derivatives. Losses of Fmoc-serine moieties from $[M - H]^-$ ions give strong peaks at *m/z* 674. Signals at *m/z* 255 (palmitic acid) and 281 (oleic acid) are diagnostic ions of fatty acyl chains, the intensities of $[R_1COO]^-$ ions are greater than those of $[R_2COO]^-$ peaks. Abundance of the peaks at *m/z* 409 ($[M - Fmoc - 88 - R_2CO - H]^-$) and 391 ($[M - Fmoc - 88 - R_2COOH]^-$) from Fmoc-16:0-18:1, as well as *m/z* 435 ($[M - Fmoc - 88 - R_2CO - H]^-$) and 391 ($[M - Fmoc - 88 - R_2COOH]^-$) from Fmoc-18:1-16:0 are significantly high (47). Because of the presence of significantly abundant $[M - Fmoc - 88 - R_2CO - H]^-$ and $[M - Fmoc - 88 - R_2COOH]^-$ fragment ions in conventional LSI mass spectra of Fmoc GPS species, the feasibility of identifying composition and location of fatty acid chains by conventional mass spectrometry of Fmoc GPS, after reversed-phase HPLC separation, has been established (47).

Figure 9 shows the conventional LSI mass spectrum of Fmoc derivative of GPS species from rat kidney. The abundant peak at *m/z* 1032 is the deprotonated molecule of Fmoc GPS 38:4. Anions at *m/z* 810 and 724 are formed by losses of Fmoc and Fmoc-serine moieties, respectively. Ions arising at *m/z* 303 ($[R_2COO]^-$; arachidonic acid), *m/z* 283 ($[R_1COO]^-$; stearic acid), *m/z* 437 ($[M - Fmoc - 88 - R_2CO - H]^-$) and *m/z* 419 ($[M - Fmoc - 88 - R_2COOH]^-$) indicate both composition and location of fatty acyl chains in this species. This lipid is identified as Fmoc GPS 18:0-20:4 (28). The other three minor species, corresponding to the weak deprotonated molecules at *m/z* 1004, 1008 and 1010, are tentatively determined as Fmoc-GPS 36:4 (16:0-20:4), -GPS 36:2 (18:1-18:1 or/and 18:0-18:2) and -GPS 36:2 (18:0-18:1), based on $[M - Fmoc - 88]^-$ fragments at *m/z* 695, 699 and 701. The fragmentation pattern of high-energy CID spectra of Fmoc GPS species is identical to that of high-energy CID of underivatized GPS species (47,48) (see Table 3).

DISCUSSION

The low- and high-energy CID of protonated and deprotonated molecules from aminophospholipids produce a wealth of information on the structure of molecular species. Tandem mass spectra obtained by low-energy CID of $[M - H]^-$ ions from aminophospholipids contain anions which are useful for characterizing polar head moieties and fatty acid chains esterified in molecular species. High-energy CID of both $[M + H]^+$ and $[M - H]^-$ ions from aminophospholipids yields numerous product ions, which are structurally informative for identifying polar heads as well as the composition and location of fatty chains in molecular species.

The negative ion LSI or ESI CID tandem mass spectrometry is commonly used in structurally determining aminophospholipid molecular species. A decision on the position of fatty acyl residues in unknown 1,2-diacyl aminophospholipid molecular species can be made based on a general rule for CID mass spectra of GPE and GPS that the intensity ratio of carboxylate anions ($[R_2COO]^-/[R_1COO]^-$) > 1 for GPE (45,47); and ($[R_2COO]^-/[R_1COO]^-$) < 1 for GPS (47,48) reflects the location of fatty acyl chains esterified. Therefore, the reproducibility of $[R_2COO]^-$ and $[R_1COO]^-$ ions in intensity, which are produced by CID of $[M - H]^-$ precursors, is essential in the structural determination of molecular species. Data presented in this study and others suggest that the kinetic energy of precursor ions ($[M - H]^-$) is presumably associated

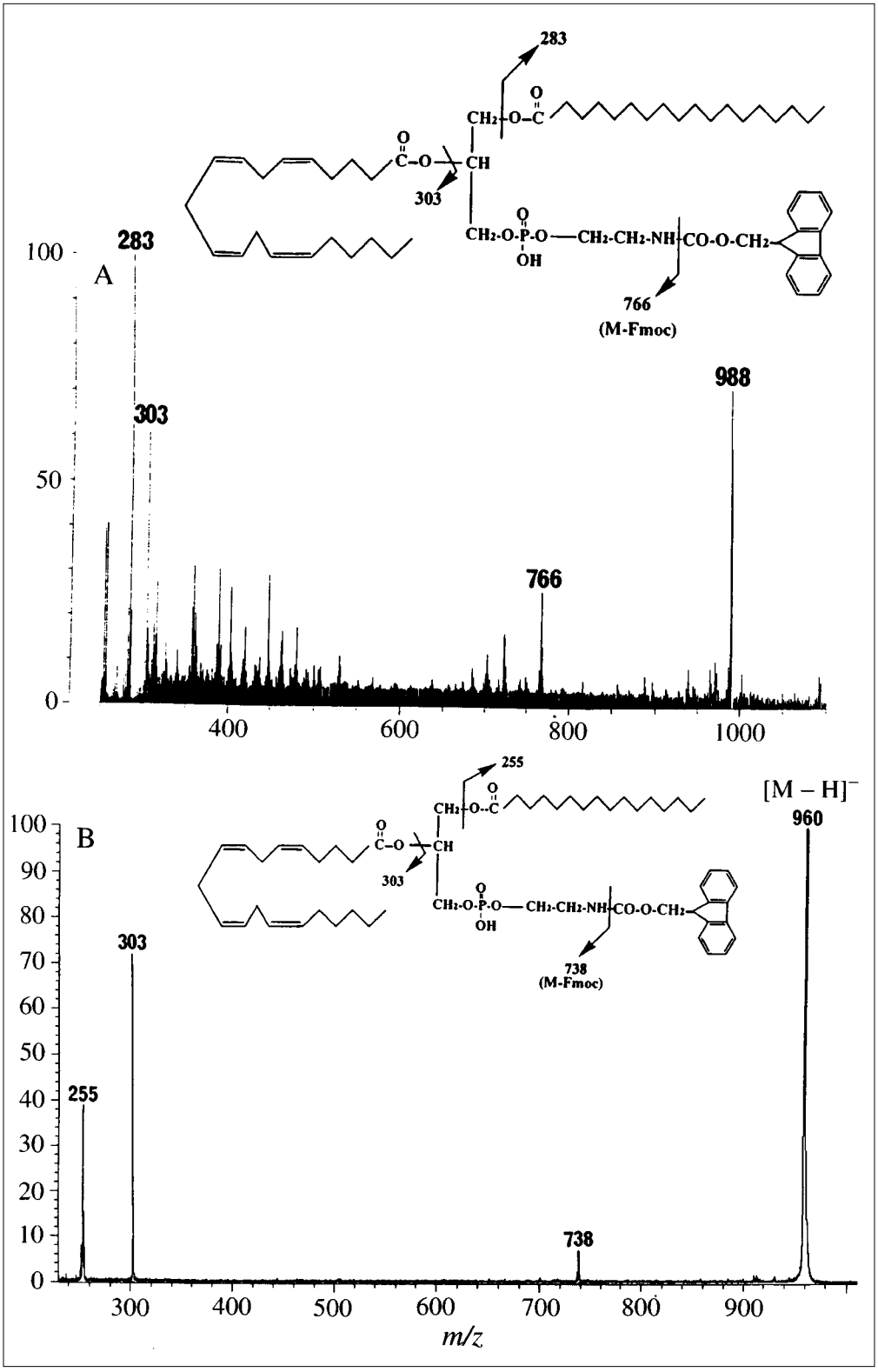


FIG. 7. Negative ion LSI mass spectrum of Fmoc derivative of GPE 18:0-20:4 (A) and high-energy CID spectrum of deprotonated molecule of reversed-phase HPLC purified-Fmoc derivative of rat kidney GPE 16:0-20:4 (B).

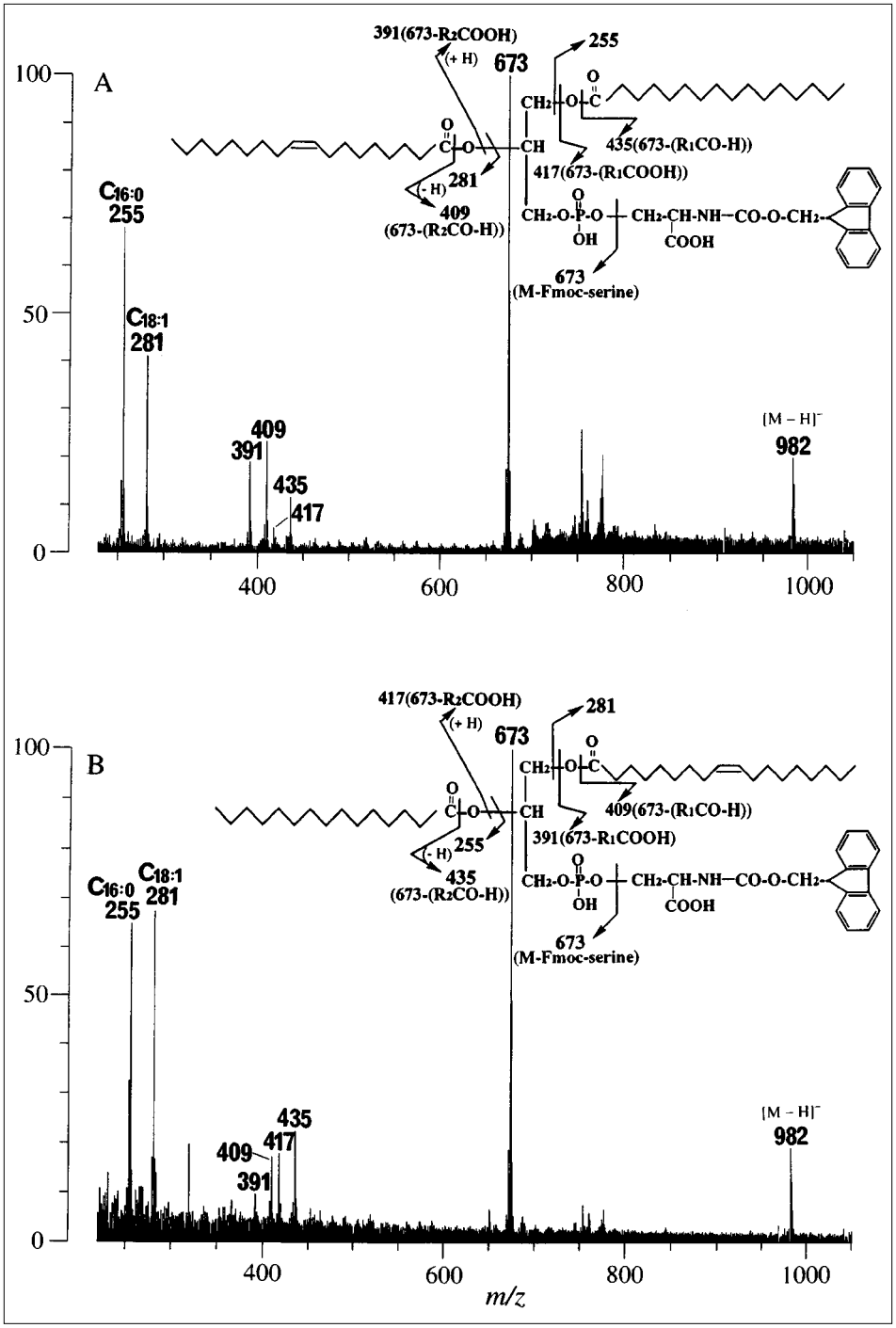


FIG. 8. Negative ion LSI mass spectra of Fmoc derivatives of GPS 16:0-18:1 (A) and GPS 18:1-16:0 (B).

with the abundance of carboxylate anions derived from them. For example, CID mass spectra of $[M - H]^-$ ions from various GPE species were produced by (i) the electric-magnetic sector coupled to low- or high-energy CID (spectra of GPE 18:0-20:4 and GPE 16:0-22:6 shown in References 47 and 48 cited in the paper); and (ii) the quadrupole mass analyzer combined with low-energy CID (spectra of GPE 18:0-22:6 shown in Refs. 37 and 26, as well as spectra of GPE 18:0-20:4 shown in Refs. 38 and 45 cited in the study) experiments, respectively. But the intensity of carboxylate anions produced by the former (47,48) rather than the latter (37,26) fully meet the general rule (45) described above. It could be explained by the fact that the kinetic energy of ion source-produced precursor ions transmitted through the electric-magnetic sector is different from that of some precursor ions transmitted through the quadrupole mass analyzer. The kinetic energy di-

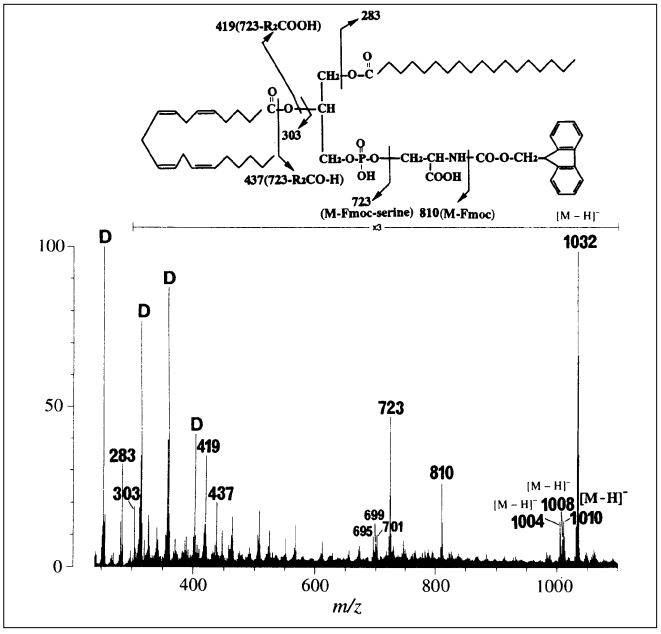


FIG. 9. Negative ion LSI mass spectrum of Fmoc derivatives of rat kidney GPS. D peaks are due to diethanolamine matrix used.

versity of ion source-produced precursor ions transmitted through the quadrupole mass analyzer might lead to the intensity irreproducibility of carboxylate anions derived from the precursors. The ESI CID method exhibits remarkable superiority in analyzing structures of aminophospholipids at the picomole levels within minutes. The electric-magnetic sector coupled to quadrupole mass analyzer or electric-magnetic sector mass spectrometers have the advantage of the capability of properly determining the location of fatty acid chains in molecular species. The combination of tandem mass spectrometry should be taken into account in interpreting CID mass spectra of molecular species of aminophospholipids. The three sorts of tandem mass spectrometers exhibit the capability of identifying the structure of various underivatized Lysophospholipids, such as LysoPE, Plasmalogen LysoPE and LysoPE regioisomers.

An approach has been developed for the structural analysis of aminophospholipid species as Fmoc derivatives in order to meet the requirements of both quantitative determination of classes and molecular species by HPLC coupled to chromophore detection and full characterization by mass spectrometry of aminophospholipid derivatives. Negative ion LSI mass spectrometry of Fmoc derivatives of GPE and GPS yields abundant $[M - H]^-$ ions and informative fragment ions which are useful for identifying structures of molecular species. High-energy CID mass spectra of the derivatized species exhibit fragmentation modes identical to those of high-energy CID of underivatized GPE and GPS. The low limit of producing a useful conventional and CID mass spectrum of the Fmoc derivatives is approximately 2 and 30 nanograms, respectively. Furthermore, a novel derivative of animophospholipid, ethyltriphenyphosphonium (ethyl-TPP) of GPS, has been prepared, and the preliminary result shows that CID of LSI-generated $[M]^+$ ion of ethyl-TPP GPS 16:0-16:0 not only provide information on the composition and location of fatty acyl chains but also allow structures of esterified-fatty acid to be identified. Further studies on HPLC separation and CID fragmentation of ethyl-TPP derivatives of aminophospholipids are in progress.

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Analysis of Hydroxy and Keto Cholesterols in Oxidized Brain Synaptosomes

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ABSTRACT: A rapid method for the simultaneous determination of cholesterol and its oxidation products as well as α-tocopherol and tocopherolquinone in brain subcellular fractions is described. The samples are saponified and extracted with hexane. It is not necessary to remove cholesterol in the sample before analyzing for oxysterols. The hexane extract can be used for the assay of cholesterol compounds by capillary gas chromatography and tocopherol compounds by liquid chromatography using a procedure reported previously. Oxidation of synaptosomes by a mixture of Fe^{2+} plus ascorbate resulted in the production of 7-keto-, 7α-hydroxy-, 7β-hydroxy-, and 5α,6α-epoxycholesterols. The identities of these products were confirmed with gas chromatography/mass spectrometry. Cholesterol oxidase treatment did not result in the formation of any of the above compounds. Thus the types and amounts of the products of oxidation of cholesterol were dependent upon the oxidizing agent. Extraction of the oxysterols under milder conditions without saponification using sodium dodecyl sulfate cannot be used since such treatment results in low recovery of oxysterols. Oxidation of synaptosomes by low concentrations of ferrous iron and ascorbate resulted in (i) low levels of oxidation of cholesterol which could be followed by estimating the production of oxysterols and (ii) oxidation of a substantial percentage of α-tocopherol. The proposed procedure will be useful in monitoring the oxidation of small quantities of membrane cholesterol *in vitro*.

Lipids 32, 101–107 (1997).

Cholesterol and phospholipids in membranes are known to undergo peroxidation which can result in structural and functional damage. Numerous oxidation products of cholesterol produced under a large variety of conditions have been identified (1). The majority of the known cholesterol oxidation products are biologically active as cytotoxic, atherogenic, mutagenic, carcinogenic, or enzyme inhibitory substances (2). Therefore, it is important to monitor the production of oxysterols during biological oxidative stress. However, little, if anything, is known about the formation of oxysterols in brain under conditions of free radical-induced oxidative stress. The biological effects of specific oxysterols upon brain function can be determined only after knowing the types and amounts of oxysterols formed in brain during peroxidative reactions. A capillary gas chromatographic (GC) method for the determination of oxysterols in brain subcellular fractions is described in this paper.

Our laboratory has been investigating the relationship between consumption of the lipid antioxidant vitamin E and oxidation of membrane cholesterol during the *in vitro* oxidation of biological samples. The experimental procedure consists of incubation of subcellular fractions from brain with oxidizing agents followed by determination of the decreases in concentrations of α-tocopherol and cholesterol and the increases in concentrations of the respective oxidation products. Only small quantities of subcellular fractions are usually available for such experiments. Hence, a method was needed for the determination of tocopherol compounds, cholesterol, and oxysterols within the same sample. We have developed a simple and rapid procedure for such analyses, and the details are reported here. A GC method for the analysis of cholesterol oxidation products in lipoprotein samples has been proposed by Addis *et al.* (3). This procedure involves extraction of lipids with chloroform plus methanol, saponification of the extracted lipids for 18–20 h at room temperature, and extraction of the unsaponified compounds with ether. This is then followed by derivatization and GC analysis. Sevanian *et al.* (4) have used GC and liquid chromatography (LC) coupled with mass spectrometry (MS) for determining oxysterols in human plasma samples. Their method also involves several steps. Determination of cholesterol oxidation products by isotope dilution–MS also has been published (5). The latter techniques require expensive equipment that is not readily available in most laboratories. The procedure used in this report is simpler and faster and involves saponification followed by a single solvent extraction step. One portion of the extract is used for determining cholesterol and its oxidation products by the procedure proposed in this paper. Another portion of

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Abbreviations: BHT, butylated hydroxytoluene; GC, gas chromatography; HPLC, high-performance liquid chromatography; LC = liquid chromatography; MS, mass spectrometry; TMCS, trimethylchlorosilane. Trivial names of sterols: cholest-5-ene-3β,7α-diol = 7α-hydroxycholesterol; cholest-5-ene-3β,7β-diol = 7β-hydroxycholesterol; 3β-hydroxycholest-5-ene-7-one = 7-ketocholesterol; 5,6,α-epoxy-5α-cholestan-3β-ol = 5α,6α-epoxycholesterol.

the extract can be utilized for determination of tocopherol and its quinone by a LC method (6). The data in this report focus primarily on the application of this technique to monitor free radical-induced oxidation of cholesterol in rat brain synaptosomes.

EXPERIMENTAL PROCEDURES

Chemicals. The chemicals used were of reagent-grade purity from standard sources. Solvents were high-performance liquid chromatography (HPLC) grade from Fisher Scientific (Pittsburgh, PA). Other special chemicals used were purchased from the following sources. Cholesterol oxidase (EC1.1.3.6) from *Cellulomonas* was purchased from Sigma Chemical Company (St. Louis, MO). The hydroxy- or ketocholesterols, 5α-,6α-epoxide and cholesterol (Sigma grade: standard for chromatography) were obtained from Sigma Chemical Company, or Research Plus Inc. (Bayonne, NJ). The 5β-,6β-epoxide of cholesterol was from Steraloids, Inc. (Wilton, NH). Regisil with 1% trimethylchlorosilane (TMCS) was used for silylation of the sterols and was purchased from Regis Technologies Inc. (Morton Grove, IL). Absolute ethanol was obtained from Midwest Solvents Company (Pekin, IL) and was redistilled prior to use. Ferrous sulfate heptahydrate from Sigma Chemicals was used as the source of ferrous iron. Most of the reagent grade chemicals were from Sigma Chemicals. The DB1 column was from J&W Scientific (Folsom, CA).

Biochemical assays. Concentration of total protein was determined by the Lowry technique as modified by Markwell *et al*. (7).

Extraction of analytes from sample. The procedure was essentially the same as published earlier (6). Briefly, 2 mL ethanol containing 0.025% (wt/vol) butylated hydroxytoluene (BHT) and 0.1 mL of 30% (wt/vol) ascorbic acid were pipetted into tubes containing samples for analyses. The mixture was heated at 60°C for 30 min after the addition of 1 mL of 10% potassium hydroxide solution. Tubes were cooled, and 2 mL of water was added followed by 2 mL of hexane containing 0.025% (wt/vol) BHT. Cholesterol, α-tocopherol, and their oxidation products were extracted into the hexane phase by vortexing for 1 min.

One portion of the hexane phase (usually 0.5 mL) was separated out and used for the analysis of cholesterol and oxysterols. Samples containing high levels of cholesterol could be analyzed with smaller volumes of the hexane extract whereas larger volumes had to be used with samples such as mitochondria containing smaller amounts of cholesterol. The extract was evaporated down under a stream of nitrogen, or the solvent was removed under vacuum at 60°C. The residue was mixed with 100 µL Regisil [*bis* (trimethyl silyl) trifluoroacetamide plus 1% trimethylchlorosilane] as derivatizing agent and 25 µL pyridine and then heated at 60°C for 1 h. The silylated cholesterol and its oxidation products were analyzed by a capillary GC method: Hewlett-Packard 5880 GC with autosampler (Palo Alto, CA), injection port 230°C, flame-ionization detector 280°C, splitless injection; DB1 column, 30 meters × 0.324 mm i.d., film thickness 0.25 µm; temperature programming—oven temperature initial 50°C, initial time 3 min, program rate 30°C per min, final value 290°C, second program rate 1°C per min, final temperature 300°C and keep at 300°C for 5 min.

The second portion of the extract was used for assays of tocopherol compounds using the HPLC procedure reported earlier (6).

Identification of cholesterol oxidation products by GC/MS. The mass spectral analyses were performed on a Kratos MS-25 GC/MS (Kratos Analytical, Ramsey, NJ). Electron impact (EI) mass spectra were acquired with an electron ionization potential of 70 eV and ion source temperature of 220°C. The conditions of derivatization and GC were the same as described in the above paragraph.

Preparation of subcellular fractions from rat brain. Four-month-old, male Fisher 344 rats were used. The subcellular fractions were isolated from cerebral hemispheres by standard centrifugation methods (8). Brain tissue was homogenized in 10 vol of ice-cold isolation medium containing 0.32 M sucrose, 10 mM HEPES, and 1 mM EDTA at pH 7.4, using a glass-glass homogenizer. The homogenate was centrifuged at 1300 × *g* for 3 min and the supernatant saved. The pellet was resuspended in 10 mL of the isolation medium, rehomogenized, and centrifuged at 1300 × *g* for 3 min. The pooled supernatants were centrifuged at 17,000 × *g* for 10 min to get the crude mitochondrial fraction. The resulting pellet was resuspended in 15 mL of isolation medium. Half of this suspension was layered over 11 mL of 7.5% (wt/vol) Ficoll medium which had been layered over 11 mL of 10% (wt/vol) Ficoll medium. The tubes were centrifuged in a Beckman SW 28 rotor (Fullerton, CA) at 99,000 × *g* for 45 min. The fraction at the interface between the two Ficoll solutions was removed, diluted 1:5 with isolation medium, and centrifuged for 10 min at 17,000 × *g* to isolate the synaptosomes. The purity of the synaptosomal fraction was tested by electron microscopy and also by estimating the activities of the marker enzyme Na-K-ATPase (9). The activity of the ATPase was usually enriched two- to three-fold in synaptosomes compared with the crude homogenate. The synaptosomes isolated are metabolically active and have been used in studies of transmitter uptake and release. Even though we used only the synaptosomes in this report, other subcellular fractions such as mitochondria and myelin also can be isolated using this procedure.

Protocol for incubations. Synaptosomes were incubated in a medium simulating extracellular fluids or plasma and contained: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium phosphate, 10 mM glucose and a pH of 7.4. HEPES (10 mM) was also added for additional buffering capacity. The oxidizing agents such as ferrous salt and ascorbate were added and the tubes incubated. When higher levels of oxidation were desired, the concentrations of oxidants and the times of incubation were increased. After incubation, the tubes were placed on ice. The reaction mixture was then cen-

trifuged for 30 min at 17,000 rpm in a Sorvall (Dupont, Hoffman Estates, IL) SS 34 rotor (35,000 × *g*) to sediment the synaptosomes. The sedimented fractions were then analyzed for various components.

In a few cases, brain subcellular fractions were incubated with cholesterol oxidase. Typically, samples of synaptosomes (500 μg of protein in a total volume of 2 mL) were incubated at 37°C for 2 h in the buffer described above in the presence of either 0.15 or 0.3 units per mL of the enzyme. After incubation, the samples were placed on ice and centrifuged immediately to sediment the synaptosomes. The pellet was then processed for the GC and LC assays.

RESULTS

A sample containing a mixture of standard oxysterols was carried through the procedure, and the chromatogram obtained is shown in Figure 1 and the corresponding reagent blank in Figure 2. It can be seen that the oxysterols of interest are separated well. The retention times in minutes of cholesterol and a few selected oxysterols were as follows: cholestane (internal standard) = 14.82; cholesterol 17.26; 7α-hydroxycholesterol (A) 16.97; 19-hydroxycholesterol (B) 17.91; 7β-hydroxycholesterol (C) 18.30; 5α,6α-epoxycholesterol (D) 18.75; 22-ketocholesterol (E) 19.19; 7-ketocholesterol (F) 20.63.

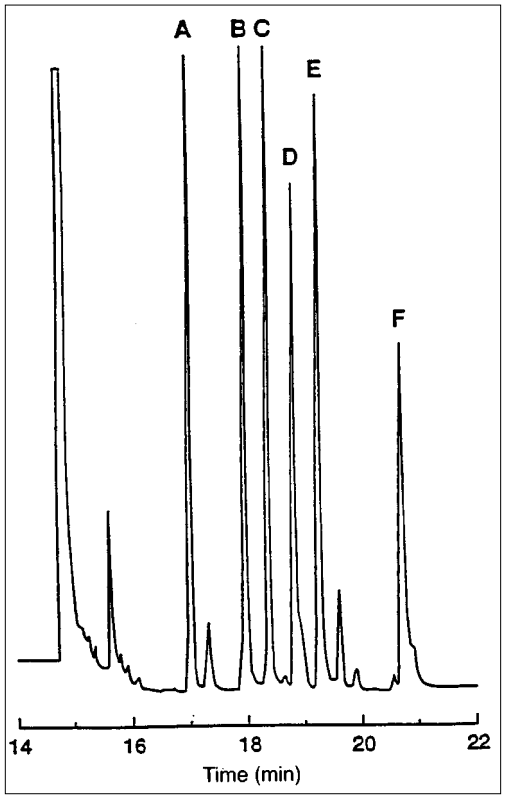

FIG. 1. Chromatogram from the analysis of a mixture of oxysterols by the current method. A mixture of standard commercial oxysterols was processed as described and analyzed by capillary gas chromatography. The peaks identified by capital letters are: 7α-hydroxycholesterol (A); 19-hydroxycholesterol (B); 7β-hydroxycholesterol (C); 5α,6α-epoxycholesterol (D); 22-ketocholesterol (E); 7-ketocholesterol (F).

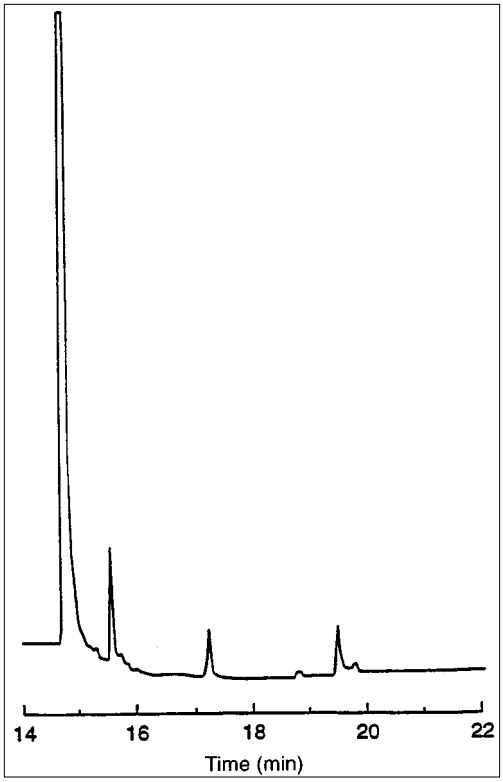

FIG. 2. Chromatogram from the analysis of a mixture of reagents used in the proposed technique. This figure serves as a reagent blank chromatogram for Figure 1.

Samples of synaptosomes were obtained from the cerebral hemispheres of four-month-old rats and were incubated *in vitro* with a mixture of 25 μM Fe^{2+} and ascorbate for 3 h. Such mixtures of $Fe^{2+}$and ascorbate have been used as sources of hydroxyl radicals in numerous investigations. The chromatogram obtained after processing the sample is given in Figure 3. The corresponding chromatogram obtained after processing a control sample of synaptosomes is shown in Figure 4. Even though many oxysterols have been reported to exist in biological systems under various conditions, peroxidation of synaptosomes resulted in the production of only a few oxysterols. The oxysterols observed chromatographically were: 7α- and 7β-hydroxycholesterol, 7-ketocholesterol and 5α,6α-epoxycholesterol. The 5β,6β-epoxycholesterol was also probably formed under the same conditions. However, we could not demonstrate this consistently.

In some cases it may be desirable to avoid saponifying the sample and extract the cholesterol derivatives using milder techniques to prevent oxidation of membrane components. One of the recommended procedures for extraction of tocopherols from biological samples without saponification involves the use of SDS to disrupt the membrane (10) . An experiment utilizing this method for extraction was performed. The final

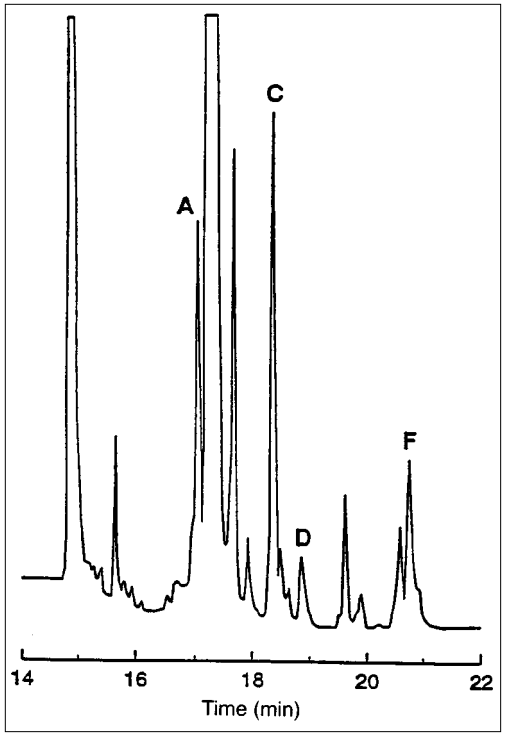

FIG. 3. Chromatogram from the analysis of a sample of synaptosomes that was incubated in the presence of a mixture of Fe^{2+} and ascorbate (25 μM each). Rat brain synaptosomes were isolated and incubated for 3 h with a mixture of Fe^{2+} and ascorbate. The cholesterol and the oxysterols produced were assayed by the recommended method.

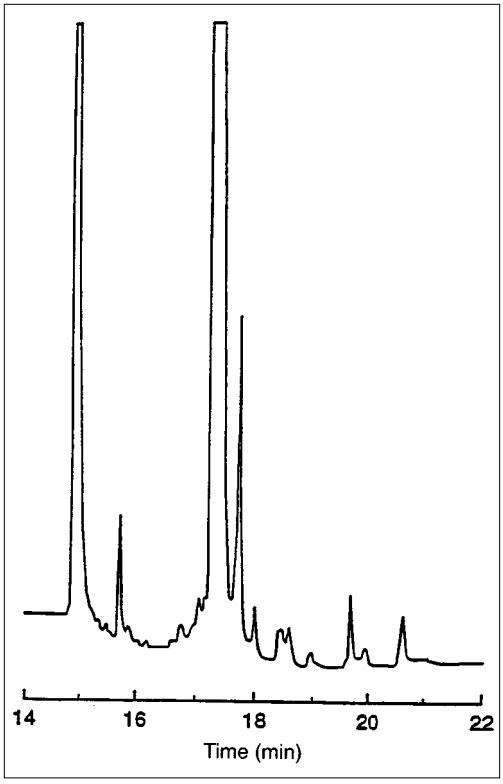

FIG. 4. Chromatogram from the analysis of a sample of synaptosomes that was incubated for 3 h with no oxidant. This chromatogram serves as control for Figure 3.

SDS concentrations used were either 0.04 M or 0.1 M. The results of this study are shown in Table 1. Substantial amounts of the oxysterols remain unextracted in the aqueous phase, causing a reduction in recoveries. In fact, higher concentrations of SDS in the medium resulted in lower recoveries of the oxidized cholesterol compounds. Thus, even though solvent extraction in the presence of SDS has been used successfully for the isolation of tocopherols, it is not suitable for quantitative extraction of oxysterols. This is not surprising since oxysterols are more water-soluble than cholesterol itself and detergents could decrease the recovery of these compounds.

The applicability of the method for quantitative analysis of oxysterols was also tested. When 4 to 30 picomoles of the sterol derivatives were injected on the column, the standard curves obtained were linear within limits of experimental error. The correlation coefficients were usually higher than 0.99. This was true for all cholesterol derivatives shown in Figure 1. The percentage recoveries of the various cholesterol derivatives added to samples of synaptosomes were as follows: 7α-hydroxycholesterol = 87 ± 3 (SD); 7β-hydroxycholesterol = 98 ± 1.7; 7-ketocholesterol = 97 ± 4.6; 22-ketocholesterol = 88 ± 4.2; and 5α,6α-epoxycholesterol = 80 ± 3.6. The lower recovery of the epoxide may reflect the instability of this compound during sample processing and chromatography. The detection limits (expressed as picomoles injected on the capillary column) for the various compounds were as follows: 7-ketocholesterol = 2, epoxide = 1, and the hydroxycholesterols = 0.5. Sevanian *et al.* (4) have reported the detection limits to be in the range of 2.5 to 5 picomoles, and the two methods are quite comparable in this respect.

TABLE 1
The Effect of SDS upon the Extractability of Oxysterols

	7α-Hydroxycholesterol			7-Ketocholesterol			5α,6α-Epoxycholesterol		
Concentration of SDS	Amount added	Amount recovered	Percentage recovery	Amount added	Amount recovered	Percentage recovery	Amount added	Amount recovered	Percentage recovery
0.04 M	5.46[a]	2.33	43	6.86	4.4	64	6.21	5.4	87
0.1 M	5.46	0.94	17	6.86	2.6	38	6.21	4.2	67

[a]The values for amount added and recovered are in nanomoles. The various amounts of oxysterols were extracted and processed for assays in the presence of the two levels of SDS. Quantitation was done by capillary gas chromatography.

One of the advantages of this procedure is that the bulk of the cholesterol in the samples need not be removed prior to the chromatographic determination of oxysterols. The effect of cholesterol content of the sample upon the reliability of the method was tested. This was done by adding different amounts of cholesterol to oxidized synaptosomes and then assaying for oxysterols. Samples of synaptosomes (600 μg of protein) were oxidized with mixtures of Fe^{2+} and ascorbate. The synaptosomal sample was expected to contain about 200 nanomoles of cholesterol. Known amounts of cholesterol, either 194 or 388 nanomoles, were added to some of the tubes, and the oxysterols were assayed by the recommended method. The results showed that the concentrations of oxysterols produced during the oxidations were not significantly different, irrespective of the amount of cholesterol present in each tube. Thus, the added cholesterol did not get oxidized during the sample processing and give rise to erroneously high levels of oxysterols. Interestingly, we found that the levels of oxysterols in control unoxidized samples of synaptosomes were quite low; the levels were lower than those found in standard cholesterol that had been kept in the laboratory for several years. In any case, the method provides reliable data on oxysterol concentrations in brain subcellular fractions without the need for cholesterol separation.

The aim of the next experiment was to compare the results of analysis of oxidized synaptosomal samples by the current method with those obtained by the method of Addis *et al*. (3). In this experiment, the synaptosomal samples were oxidized with a mixture of Fe^{2+} and ascorbate. The results are given in Table 2. It can be seen that the two methods provide comparable concentrations of oxysterols.

Many investigators have used cholesterol oxidase to alter membrane cholesterol concentrations. After incubations with cholesterol oxidase the percent oxidation of membrane cholesterol was: 2.4 ± 1.2 and 16.2 ± 1.5 with 0.15 or 0.3 units per mL of the enzyme, respectively. The α-tocopherol concentration was not changed during treatment with cholesterol oxidase. The gas chromatogram of oxysterols contained two peaks whose retention times were close to 19-hydroxycholesterol. Since the main product of cholesterol oxidation with the oxidase enzyme is the 3-keto derivative, these peaks may have been derived from this compound. Since the focus of our investigations is the analysis of oxysterol production during free radical-induced peroxidation, we did not attempt to characterize and identify these unknown peaks. It is interesting to note that none of the peroxidation products such as the 7-hydroxy or 7-keto derivatives were formed during treatment with cholesterol oxidase.

To show the utility of this technique, we subjected a few samples of synaptosomes to oxidation in the presence of a mixture of Fe^{2+} and ascorbate (10 μM each). The data are shown in Table 3. Firstly, it can be seen that no significant change can be observed in the levels of total cholesterol as a result of oxidation. If cholesterol oxidation was followed simply by examining whether there was a decrease in cholesterol concentration after the *in vitro* treatment, one would have concluded erroneously that cholesterol was not oxidized. However, data on the production of cholesterol oxidation products clearly show that a mixture of Fe^{2+} and ascorbate causes oxidation of membrane cholesterol. Secondly, the pattern of distribution of the oxidation products of cholesterol is characteristic of oxidations induced by hydroxyl radicals. Thirdly, even though little cholesterol was consumed, a substantial portion of α-tocopherol was oxidized. Hence, α-tocopherol is indeed acting as a chain-breaking antioxidant, preventing the oxidation of substantial amounts of membrane cholesterol. Table 3 represents the type of data on cholesterol and α-tocopherol and their oxidation products that can be obtained from the analysis of a single extract from the subcellular fraction. It can be seen that the proposed method provides a significant amount of information on the oxidation of the two important membrane components, cholesterol and tocopherol.

The cholesterol oxidation products were identified by GC/MS. Both mass spectral fragmentation patterns and GC retention times of the compounds as their trimethylsilyl-ether derivatives were compared to authentic standards and found to be the same. The 7α- and 7β-hydroxycholesterols had a molecular ion of *m/z* 546, a base peak at *m/z* 456, and an ion fragment at *m/z* 366. The 7-ketocholesterol had a molecular ion and base peak at *m/z* 472 and ion fragments at *m/z* 382 and 387. The 5α,6α-epoxycholesterol had a molecular ion of *m/z* 474 and ion fragments at *m/z* 459, 384, and 386. All of the compounds had the characteristic retention times noted earlier. The mass spectra of oxidized samples from synaptosomes matched with the mass spectra of standards at the specific retention times. It was mentioned above that oxysterol concentrations were very low in samples of synaptosomes in-

TABLE 2
Comparison of Oxysterol Concentrations in Control and Oxidized Synaptosomes After Analysis by the Current Technique or the Procedure of Addis *et al.* (3)[a]

	Cholesterol		7α-Hydroxycholesterol		7β-Hydroxycholesterol		7-Ketocholesterol		5α,6α-Epoxycholesterol	
	Current method	Addis *et al.* (Ref. 3)	Current method	Addis *et al.* (Ref. 3)	Current method	Addis *et al.* (Ref. 3)	Current method	Addis *et al.* method	Current method	Addis *et al.* method
Control	352 ± 3*	345 ± 5	—**	—**	0.58 ± 0 01	0.60 ± 0.01	—**	—**	—**	—**
Iron + ascorbate	312 ± 7	306 ± 8	3.4 ± 0.03	3.9 ± 0.2	5.1 ± 0.3	5.3 ± 0.1	6.8 ± 1.3	7.4 ± 0.1	1.6 ± 0.2	1.6 ± 0.1

[a]Samples of synaptosomes (750 μg total protein) were incubated for 4 h with or without a mixture of Fe^{+2} and ascorbate (100 μM each). The samples were processed as described and the cholesterol and its oxidation products determined by capillary gas chromatography. *Mean ± SD (n = 3). The values refer to the concentration in nanomoles per mg of synaptosomal protein. ** Concentrations were below detection limits.

TABLE 3
Production of Oxysterols When Synaptosomal Samples Were Incubated with Low Concentrations of a Mixture of Fe^{+2} and Ascorbate[a]

Concentration of Fe^{+2} and ascorbate	Cholesterol	7α-Hydroxy cholesterol	7β-Hydroxy cholesterol	7-Keto cholesterol	5α,6α-Epoxy cholesterol	α-Tocopherol	α-Tocopherol-quinone
0	325 ± 4*	0.9 ± 0.1	0.5 ± 0.03	—**	0.33 ± 0.02	0.281 ± .004	0.025 ± .0012
10 μM	309 ± 9	1.5 ± 0.2	2.2 ± 0.06	1.75 ± 0.03	0.68 ± 0.05	0.129 ± .002	0.073 ± .006

[a]Samples of synaptosomes (750 μg total protein) were incubated for 3 h with or without a mixture of Fe^{+2} and ascorbate. After incubations the synaptosomes were sedimented, processed, and analyzed by capillary gas chromatography and tocopherol and tocopherolquinone by high-performance liquid chromatography. *Mean ± SD (n = 3). The values refer to the nanomoles present per mg of total protein. ** Concentration was below detection limit.

cubated with no oxidizing agent. We could not observe any oxysterols by GC/MS under these conditions.

DISCUSSION

One of the main advanatages of this method is the use of a single solvent extract for the determination of tocopherol, cholesterol, and their oxidation products. In addition, the method also allows the GC determination of cholesterol as well as oxysterols in biological samples. Even though the concentration of cholesterol is much higher than those of the oxidation products, it is not necessary to separate the bulk of cholesterol prior to the GC assay. A part of the extract can be used for the LC determination of tocopherol using methodology which has been published (6,11).

Oxidation of cholesterol would be expected to yield hydroperoxy derivatives. Biological tissues contain peroxidases which would catalyze the reduction of these hydroperoxides to the corresponding alcohols. A study by Thomas *et al*. (12) has shown that the phospholipid hydroperoxide glutathione hydroperoxidase enzyme which is present in many membranes can hydrolyze cholesterol hydroperoxides. Hence the levels of cholesterol hydroperoxides in tissues would be expected to be small, and the hydroxylated compounds would serve as reliable indicators of cholesterol oxidation in biological samples. This was our rationale for developing the current method for assaying hydroxylated and keto derivatives of cholesterol. Methods for the separation and quantitation of cholesterol hydroperoxides have been reported. For example, Korytowski *et al*. (13) used HPLC with electrochemical detection to quantitate cholesterol hydroperoxides produced by photodynamic action. Another HPLC method for the assay of four oxidation products of cholesterol from muscle and liver tissue has been reported by Csallany *et al.* (14). The procedure involved lipid extraction with chloroform/methanol and normal phase LC with ultraviolet detection. Even though these procedures are useful, the current technique involving analyses of oxysterols by capillary GC will have the ability to separate many more of the oxysterols in the same chromatographic run.

The method proposed by Sevanian *et al*. (4) involves GC or HPLC with MS. The processing of samples is quite lengthy and involves extraction of lipids, preliminary purification using column chromatography, and alkaline saponification followed by GC analysis. The GC separation of cholesterol oxidation products is somewhat similar to the procedure that we have used. As expected, the elution order of the various oxidation products in the procedure of Sevanian *et al*. (4) is identical to that observed by us.

The experiments with cholesterol oxidase show that the procedure can be used for following the mechanism of different types of oxidation of cholesterol in biological membranes. The chromatographic profile of cholesterol oxidation products after cholesterol oxidase treatment was qualitatively different from that from incubations with Fe^{2+} and ascorbate. It is interesting to note that the 7-position of cholesterol is susceptible to oxidation and that hydroperoxy, oxy, and hydroxy derivatives in this position are formed quite frequently as a result of autoxidation. Cholesterol derivatives which are hydroxylated in the side chain or 5,6-epoxides can also be formed as minor products of autoxidation of cholesterol (1). We found that significant amounts of epoxides were formed after attack of membrane cholesterol by hydroxyl radicals.

The concentrations of cholesterol in biological membranes are quite high, and it would be very difficult to follow oxidation of cholesterol by monitoring small declines in cholesterol concentrations during oxidations. However, cholesterol oxidation can be monitored by determining oxysterol concentrations as proposed in this paper. The efficiency of capillary columns can be exploited in developing chromatographic conditions to separate and analyze the various oxysterols. The proposed technique has the potential to be a useful method for the analysis of oxysterols generated *in vitro* in biological samples by a variety of oxidants.

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COMMUNICATION

The Effects of Infusion of Trieicosapentaenoyl–Glycerol Emulsion on Extravascular Lung Water During Myocardial Ischemia and Reperfusion in Dogs

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ABSTRACT: To test the effects of eicosapentaenoic acid (EPA) infusion on pulmonary edema induced by coronary ligation and reperfusion, extravascular lung water (EVLW) was measured *in situ* by the thermal-dye double indicator dilution method in dogs. In the control group of five dogs, 30 mL of a 10% soybean oil emulsion was infused through a leg vein. One hour after infusion, the left anterior descending coronary artery below the first diagonal branch was ligated for 15 min and then reperfused for 30 min. In the EPA group, six dogs were similarly treated with an emulsion of a 10% trieicosapentaenoyl–glycerol (90% pure). EVLW, pulmonary capillary wedge pressure, mean pulmonary artery pressure, mean blood pressure, and cardiac index were measured before and 15 min after coronary ligation, and 15 min and 30 min after coronary reperfusion. There were no significant differences in the hemodynamic indices between the two groups. EVLW significantly increased up to two times of baseline during coronary ligation in the control group ($P < 0.05$) and more during reperfusion ($P < 0.01$), whereas EVLW did not increase in the EPA group. In conclusion, EPA inhibited EVLW accumulation and may be useful for ameliorating one of the ischemia–reperfusion-induced complications, pulmonary edema.

Lipids 32, 109–114 (1997).

Pulmonary edema during myocardial ischemia is generally believed to result from high pulmonary microvascular pressure. However, the edema may take place even at normal pulmonary wedge pressure (1–6). Increased pulmonary microvascular permeability (1–6) is probably the cause of the edema if the pulmonary capillary wedge pressure is normal. A few mechanisms are considered for increased pulmonary vascular permeability. Among them are platelet-activating factor (6) and leukotriene B4 (LTB_4). On the other hand, reperfusion after coronary ligation also induces formidable complications such as ventricular arrhythmia, vascular damage and no-reflow, myocardial functional stunning (7), and even pulmonary edema as shown in the present study.

One of the authors (T.H.) has been developing an infusible emulsion of trieicosapentaenoyl–glycerol (EPA–TG), and his group has found that the LTB_4 production by rabbit polymorphonuclear leukocytes is markedly reduced 6 h after intravenous infusion of the emulsion (8). This finding prompted us to investigate the possibility that a prior infusion of EPA–TG might reduce microvascular permeability through the reduction of LTB_4 synthesis and hence extravascular lung water (EVLW) during coronary reperfusion. If the infusion of EPA–TG emulsion reduced EVLW, we might be able to have a new tool to control one of the complications of myocardial ischemia and reperfusion.

EXPERIMENTAL PROCEDURES

General preparation. Thirteen mongrel dogs weighing 18 ± 5 kg were anesthetized with intravenous pentobarbital (25 mg/kg) and supplemented as necessary during the experiment. Dogs were ventilated through a cuffed orotracheal tube by a Harvard animal respirator at the tidal volume of 15 mL of room air/kg and the rate of 13 to 15 breaths/min. The dogs' lungs were inflated every 15 min to minimize microatelectasis.

A 7F Swan-Ganz catheter was placed into a pulmonary artery from a femoral vein, and the position of the catheter was determined from pressure recordings. A 5F lung water catheter (Model 96B-020; Edwards Laboratory, Irvine, CA) was advanced into the descending aorta from a femoral artery. The pacing wire was placed at the right atrium and constantly paced at the rate of 120 beats/minute. Schematic illustration is shown in Figure 1.

Emulsions. A 10% emulsion of EPA–TG (90% EPA and 5% arachidonic acid) was prepared as described previously (8) with slight modifications. We used a 10% soybean oil emulsion (Intralipid; Otsuka Pharmaceutical Co., Tokyo, Japan) as a control emulsion. About 60% of the oil in the control emulsion was linoleic acid. The emulsifier of the two

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Abbreviations: EPA, eicosapentaenoic acid; EPA–TG, trieicosapentaenoyl-glycerol; EVLW, extravascular lung water; LT, leukotriene.

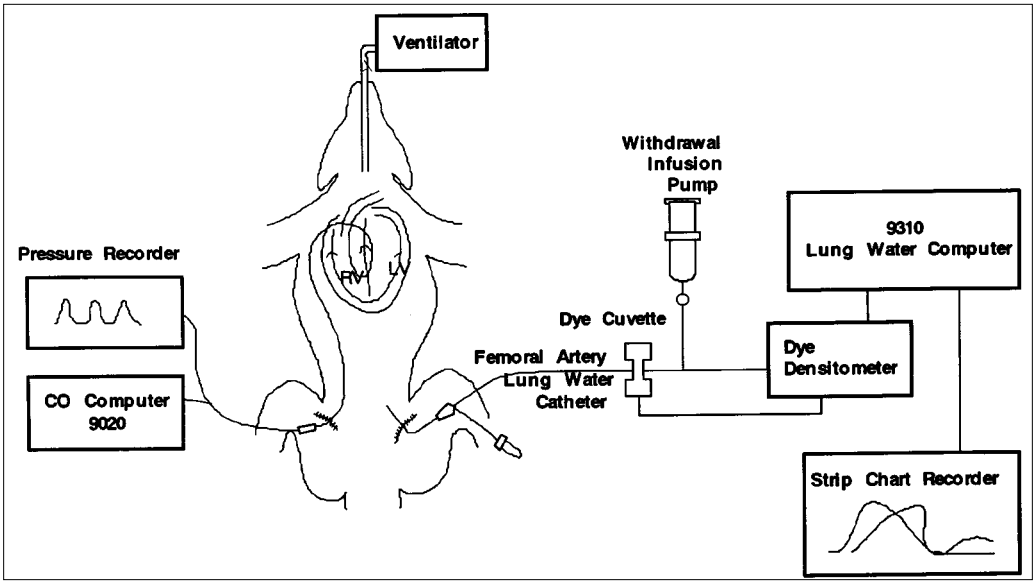

FIG. 1. Schematic illustration of measurement of hemodynamic indices and EVLW.

emulsions was egg yolk lecithin, comprising 1.2% of the total weight of emulsion (8).

Measurements. EVLW was measured by injecting 10 mL of a cold (0°C) saline containing 5 mg of indocyanine green into the right atrium through the Swan-Ganz catheter. Blood was simultaneously withdrawn by a syringe pump through a densitometer cuvette (DC-410; Waters, Rochester, MN) attached to the femoral lung water catheter. A dye signal proportional to dye concentrations was generated in the densitometer, and a thermal signal was generated in a thermistor located at the end of the lung water catheter. Both signals were fed into a computer (Model 9310; Edwards Laboratory, Santa Ana, CA) that simultaneously determined the mean transit times of the two indicators and the cardiac output. EVLW was derived as follows: EVLW = cardiac output × (mean thermal transit time minus mean dye transit time). Blood pressure was measured by a transducer connected to the femoral arterial catheter. Pulmonary arterial pressure and pulmonary capillary wedge pressure were obtained from another transducer connected to the Swan-Ganz catheter. Measurements were performed during a 10 s breath-hold at end expiration. The limb and epicardial leads in electrocardiogram were continuously monitored to evaluate heart rate and myocardial ischemia throughout the experiment.

The lung was biopsied immediately before the infusion and at 1.75 h after completion of the infusion for the measurement of EPA concentrations. Biopsied tissues were minced and rinsed with saline before analysis. The fatty acid composition of the total phospholipid fraction was analyzed by gas chromatography (a GC-14 gas chromatograph, Shimadzu, Kyoto, Japan; with a Supelcowax-10 capillary column, Supelco, Bellefonte, PA) (9).

Experimental protocol. After the dogs were treated as shown in the section of General preparation, dogs in the control group ($n = 7$) were infused with 30 mL of the soybean oil emulsion over 30 min, and those in the EPA group ($n = 6$) were done so with the EPA–TG emulsion. A suture was placed around the left anterior descending artery below the first major diagonal branch. Coronary ligation was started one hour after the infusion was completed, and the artery was ligated for 15 min. Myocardial ischemia was confirmed by the change of myocardial color from its usual red to a dusky blue, the changes in the ST segment in epicardial electrocardiogram, abnormal regional contraction in the distribution of the ligated vessel, and postmortem macroscopic examination of the heart. EVLW and hemodynamic indices were measured immediately before the lung biopsy, before and 15 min after coronary ligation, and 15 and 30 min after reperfusion as shown in Figure 2. In the control group, two dogs developed ventricular fibrillation during coronary ligation and reperfusion. One dog died, and the other could not maintain stable hemodynamics after counter shock. Therefore, the remaining five dogs were as a control group. In the EPA group, none developed ventricular fibrillation.

All experimental studies were performed along with the animal use guideline set by the National Institutes of Health and the American Physiological Society.

Statistics. Values are expressed as means ± SD. With regard to hemodynamic indices and EVLW, two-way analysis of variance was used, and intragroup comparisons with baseline were performed by Scheffe's method if analysis of variance detected significant difference. For intragroup comparisons of the fatty acid composition, paired *t*-test was used. Differences at baseline between the control and EPA groups were analyzed by unpaired *t*-test for all parameters. $P < 0.05$ was considered significant.

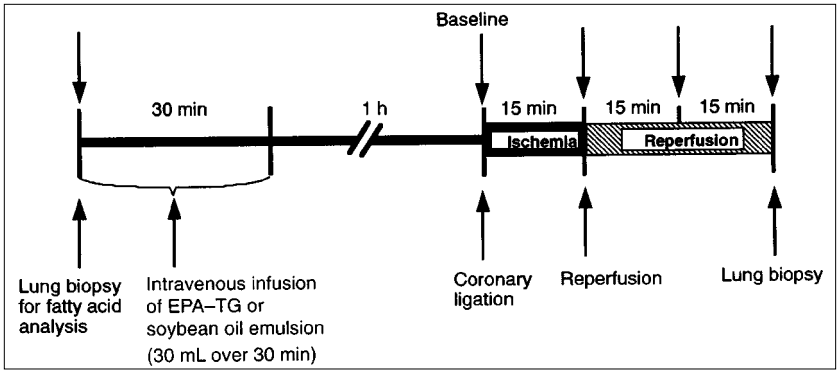

FIG. 2. Experimental protocol. Measurement (extravascular lung water, cardiac index, pulmonary capillary wedge pressure). Experiments started following the general preparation as written in the text. Abbreviation: EPA–TG, trieicosapentaenoyl–glycerol.

RESULTS

Changes in hemodynamic indices after coronary ligation and reperfusion. Pulmonary capillary wedge pressure and mean pulmonary artery pressure increased significantly after coronary ligation in both groups (Table 1). However, there were no significant differences in any measured hemodynamic indices between the control and EPA groups.

Changes in EVLW after coronary ligation and reperfusion. Except for the two control dogs that developed ventricular fibrillation, there were no serious arrhythmias in either group. There was no significant difference in EVLW between the two groups at baseline. In the control group, EVLW increased significantly after coronary ligation and reperfusion. However, EVLW did not increase significantly after coronary ligation and reperfusion in the EPA group. EVLW was significantly larger in the control group than in the EPA group after coronary reperfusion($P < 0.01$, Fig. 3).

Changes in the fatty acid composition in the lung tissue before and after infusion. As shown in Table 2, there were no significant changes in the important unsaturated fatty acids of the lung total phospholipid fraction in the control group. On the other hand, EPA increased about 2.5 times in the EPA group (Table 2).

DISCUSSION

The thermal-dye technique is a good method for EVLW measurement because an excellent correlation has been found in a few laboratories (10–12) including ours (5) between this technique and gravimetric estimates in normal and edematous lungs of animals. Therefore, we did not employ an additional method for EVLW measurement.

We chose a soybean oil emulsion (Intralipid) as the control emulsion because we had thought that the fatty acid composition of the lung would probably be constant even after

TABLE 1
Changes in Hemodynamic Indices[a]

Indices	Groups	Baseline	Coronary ligation (15 min)	Reperfusion 15 min	Reperfusion 30 min
Pulmonary capillary wedge pressure (mmHg)	Control	8.2 ± 0.8	11.2 ± 0.8[b]	8.4 ± 0.9	7.6 ± 0.9
	EPA	7.5 ± 1.0	9.8 ± 1.0[b]	7.5 ± 1.0	7.2 ± 1.2
Mean pulmonary artery pressure (mmHg)	Control	14.9 ± 0.4	17.8 ± 2.2[b]	15.7 ± 0.6	157.7 ± 1.5
	EPA	14.3 ± 1.2	16.6 ± 1.5[b]	15.0 ± 1.3	15.0 ± 1.0
Cardiac index (mL/min/kg)	Control	153 ± 71	136 ± 60	137 ± 64	137 ± 68
	EPA	130 ± 27	120 ± 47	132 ± 43	123 ± 40
Mean blood pressure (mmHg)	Control	85 ± 11	79 ± 12	80 ± 18	81 ± 20
	EPA	79 ± 8	71 ± 25	79 ± 24	72 ± 23

[a]Hemodynamic indices were measured before coronary ligation and afterward as shown in Figure 2. There were also no significant differences between the two groups in any parameters shown above by analysis of variance. Pre-infusion values of the above parameters were very similar to baseline (data not shown).
[b]$P < 0.05$ vs. baseline; EPA, eicosapentaenoic acid.

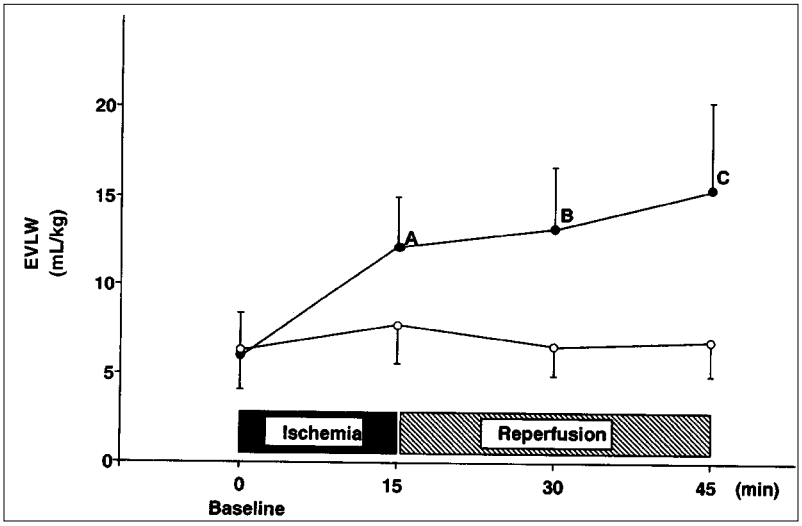

FIG. 3. Changes of EVLW. EVLW was measured at the indicated points. Pre-infusion values were very similar to baseline (data not shown). There was a significant difference between the two groups ($P < 0.01$ by ANOVA). ●, the control group ($n = 5$); ○, the EPA group ($n = 6$). The control group A: significantly different from baseline at $P < 0.05$. B: significantly different from baseline at $P < 0.01$. C: significantly different from baseline at $P < 0.001$.

soybean oil infusion. In case of rabbit polymorphonuclear leukocytes, the fatty acid composition in the total phospholipid fraction did not change 6 h after the infusion of a soybean oil emulsion (8). In fact there were no significant changes in the fatty acid composition in the control group in the present study, which suggests that this emulsion is the ideal control emulsion. If this emulsion had changed the lung phospholipid fatty acids, the comparison between the control and EPA emulsions would have become far more difficult. We showed ischemia–reperfusion induced pulmonary edema in our previous study (Izuoka, T., Takayama, Y., Sugiura, T., Taniguchi, H., Tamura, T., Kitashiro, S., Jikuhara, T., and Iwasaka, T., submitted for publication). In that experiment the accumulation of EVLW after ischemia–reperfusion in the control dogs, which had received only a small amount of saline (10 mL) before coronary ligation, proceeded exactly like the present control dogs. This finding also indicates appropriateness of the control emulsion.

The emulsifier of the two emulsions in the present study was egg yolk lecithin, the major unsaturated fatty acid of which was oleic acid (~25%). That oleic acid did not increase in the total phospholipid fraction in the lung in either group (Table 2) indicates that the two emulsions were fine enough to escape direct trapping in lung microvasculature.

In studies of oral administration of fish oil, arachidonic acid usually decreases in the phospholipid fraction to compensate increased EPA and docosahexaenoic acid (13). However, this is not always true in case of intravenous infusion of EPA (8). As shown in Table 2, arachidonic acid concentrations did not decrease in the EPA group in the present study either. The presence of arachidonic acid (5%) in the EPA–TG preparation used in the present study partly explains the sta-

TABLE 2
Changes in Important Unsaturated Fatty Acids (mol%) After Infusion of Emulsions in the Lung Phospholipid Fraction[a]

	Groups			
	Control ($n = 5$)		EPA ($n = 6$)	
Fatty acids	Preinfusion	Postinfusion	Preinfusion	Postinfusion
Oleic acid	13.2 ± 2.5	12.8 ± 1.2	12.1 ± 2.1	12.1 ± 2.0
Linoleic acid	8.6 ± 2.1	8.6 ± 0.2	7.4 ± 1.9	6.9 ± 1.7[b]
Arachidonic acid	9.3 ± 2.2	9.7 ± 1.3	10.9 ± 2.1	10.6 ± 2.9
EPA	0.67 ± 0.42	0.67 ± 0.40	0.62 ± 0.18	1.50 ± 0.41[c]
Docosahexaenoic acid	0.82 ± 0.21	0.77 ± 0.23	0.80 ± 0.25	0.84 ± 0.30

[a]Dogs were infused with 30 mL of either a 10% soybean oil emulsion (in the control group) or a 10% EPA emulsion (in the EPA group). Lung tissues were obtained before the infusion and 1.75 h after completion of the infusion. The postinfusion fatty acid composition of the total phospholipid fraction of lung was compared with preinfusion values.
[b]$P < 0.05$.
[c]$P < 0.001$. See Table 1 for abbreviation.

bility of arachidonic acid concentrations in tissue phospholipids. Long-term oral administration of fish oil appears to inhibit the elongation/desaturation process of dietary linoleic acid, which decreases the tissue arachidonic acid concentration (13). This long-term effect is not possible in a short-term experiment like ours. From these reasons, the stable arachidonic acid concentration in tissue phospholipids after EPA infusion is not surprising.

EPA is the major n-3 polyunsaturated fatty acid of fish oil. In humans, dietary fish oil supplementation has favorable effects on hyperlipidemia (14) and blood pressure in hypertensive patients (15). In fact, intervention with fish oil reduced death rates in a secondary prevention trial (16). Reduction in myocardial infarct size after coronary artery ligation has also been demonstrated in animal models given dietary fish oil supplementation (17–19). Moreover, McLennan *et al*. (20) showed that dietary fish oil prevented ventricular fibrillation following coronary ligation and reperfusion. These reports on fish oil indicate beneficial effects of fish oil during myocardial ischemia and reperfusion. However, dietary supplementation with fish oil is recommended only before the coronary event or afterward, and certainly not during the acute phase, since it takes at least a couple of weeks for fish oil to take effect. This is one of the major weak points of dietary fish oil supplementation. In this context, an infusible EPA emulsion may play its major role.

Using specially prepared dogs, Billman *et al*. (21) showed that the intravenous infusion of an emulsion of a fish oil concentrate containing free EPA and docosahexaenoic acid into those dogs prevented ventricular fibrillation induced by the exercise-induced ischemia test that would predictably develop ventricular fibrillation, whereas infusion of a soybean oil emulsion did not prevent ventricular fibrillation at all. Since the purpose of our present study was different from that of Billman *et al*. (21), our dogs were not prepared for arrhythmia. Nonetheless, it is interesting that none of the EPA-infused dogs developed ventricular fibrillation.

Since there were no significant differences between the two groups in hemodynamic indices, the difference in EVLW between the two groups indicates the difference in vascular permeability between them. In fact EVLW continued to increase during reperfusion in the control group even though pulmonary capillary wedge pressure returned to preligation levels. The substances responsible for reperfusion injury include overloaded calcium, oxygen free radicals, platelet activating factor, and eicosanoids (6,22–24), many of which increase vascular permeability. LTB_4, one of the eicosanoids, has been shown to increase pulmonary vascular permeability in the presence of polymorphonuclear leukocytes without affecting pulmonary artery and pulmonary capillary wedge pressures (25).

We have shown that infusion of EPA–TG into rabbits tended to reduced LTB_4 production by 26% in 1 h and significantly reduced it by 68% in 6 h (8). Consequently, increase of EVLW might have been prevented in the EPA group at least partly owing to the depression of LTB_4 production by EPA infusion. Because we stopped the measurement of EVLW at 30 min of reperfusion, there is a possibility that EVLW in the EPA group might have increased to the level of the control group afterward. However, that seems unlikely because LTB_4 production decrease as time passed after EPA infusion as in the case of the rabbits (8). Mechanistic studies and longer observation of EVLW remain to be investigated.

In conclusion, prior EPA infusion prevented the increase of EVLW during ischemia and in the early phase of reperfusion.

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ARTICLE

Altered Acyl Chain Length Specificity of *Rhizopus delemar* Lipase Through Mutagenesis and Molecular Modeling

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ABSTRACT: The acyl binding site of *Rhizopus delemar* prolipase and mature lipase was altered through site-directed mutagenesis to improve lipase specificity for short- or medium-chain length fatty acids. Computer-generated structural models of *R. delemar* lipase were used in mutant protein design and in the interpretation of the catalytic properties of the resulting recombinant enzymes. Molecular dynamics simulations of the double mutant, val209trp + phe112trp, predicted that the introduction of trp112 and trp209 in the acyl binding groove would sterically hinder the docking of fatty acids longer than butyric acid. Assayed against a mixture of triacylglycerol substrates, the val209trp + phe112trp mature lipase mutant showed an 80-fold increase in the hydrolysis of tributyrin relative to the hydrolysis of tricaprylin while no triolein hydrolysis was detected. By comparison, the val94Trp mutant, predicted to pose steric or geometric constraints for docking fatty acids longer than caprylic acid in the acyl binding groove, resulted in a modest 1.4-fold increase in tricaprylin hydrolysis relative to the hydrolysis of tributyrin. Molecular models of the double mutant phe95asp + phe214arg indicated the creation of a salt bridge between asp95 and arg214 across the distal end of the acyl binding groove. When challenged with a mixture of triacylglycerols, the phe95asp + phe214arg substitutions resulted in an enzyme with 3-fold enhanced relative activity for tricaprylin compared to triolein, suggesting that structural determinants for medium-chain length specificity may reside in the distal end of the acyl binding groove. Attempts to introduce a salt bridge within 8 Å of the active site by the double mutation leu146lys + ser115asp destroyed catalytic activity entirely. Similarly, the substitution of polar Gln at the rim of the acyl binding groove for phe112 largely eliminated catalytic activity of the lipase.
Lipids 32, 123–130 (1997).

Lipases (acylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that catalyze reversibly the cleavage of ester bonds of triacylglycerols to yield free fatty acids, diacylglycerols, and monoacylglycerols. Lipases are unique among the family of serine esterases in that their catalytic activity is greatly enhanced by the presence of a lipid–water interface, a phenomenon known as interfacial activation (1–3). Various lipases show strong regiospecificity and varying degrees of selectivity and stereospecificity toward different fatty acids (4–6). For industrial application, lipases with pronounced specificity (regio-, stereo-, or fatty acid specificity) are of special interest as these specificities may be exploited to produce products not obtainable by conventional chemical catalysts (7,8).

The fungus *Rhizopus delemar* (presently designated *R. oryzae*) produces extracellular lipases, one of which has been extensively characterized. The *R. delemar* lipase (*Rd* lipase) has been purified and characterized (9), a complementary DNA (cDNA) encoding the enzyme cloned and sequenced (10), and the mature (269 amino acids) and proenzyme (includes 97 amino acid propeptide) forms of the lipase expressed to high levels in *Escherichia coli* (11). *Rd* lipase, like the closely related *Rhizomucor miehei* and *Humicola lanuginosa* lipases, is termed a 1,3-specific lipase, as it exhibits strong preference for the hydrolysis of ester bonds at the *sn*-1 and *sn*-3 positions of a triacylglycerol. The crystal structure of *Rd* lipase has been solved at 2.6 Å resolution (12). The molecular architecture of the enzyme comprises a single, roughly spherical domain containing predominantly parallel β-sheets with conserved α-helices packed on either side. The active center consists of a triad of ser145, his257 and asp204, while an oxyanion hole, presumably formed in the active species by the hydroxyl and main-chain amide groups of thr83, is postulated to stabilize the tetrahedral transition state intermediate (12). In *Rd* lipase, as in all lipases studied, the catalytic center is buried beneath a surface loop or lid consisting of a short amphipathic helix (2,12). The acyl binding region of *Rd* lipase consists of a short hydrophobic trough on the enzyme surface beneath the lid. After absorption to the lipid–water interface, the lid helix is displaced. As a result of this conformational change, the active site is exposed, the oxyanion hole is formed and a large hydrophobic surface is created. This conformational change along with the composition of the lipid substrate constitutes the dominant factors in modulating lipase activities at the oil–water interface (2,12,13).

A major objective of protein engineering efforts is to improve the substrate specificity of the enzyme or create novel catalytic activities by site-directed mutagenesis. Engineering the substrate binding region may tailor the lipase to a particu-

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Abbreviations: cDNA, complementary DNA; IPTG, isopropyl-B-D-thiogalactoside; Phe, phenylalanine; *Rd, Rhizopus delemar*; TB, tributyrin; TC, tricaprylin; TL, trilaurin; TO, triolein; Tris, *tris*(hydroxymethyl)aminomethane; val, valine.

lar substrate, alter enantioselectivity, or even broaden the substrate specificity of the enzyme. Joerger and Haas (14) initiated studies to alter the chain length selectivity of *Rd* lipase through site-directed mutagenesis. The authors utilized the crystal structure of *R. miehei* lipase (15,16) to identify corresponding residues in the substrate binding region of *Rd* lipase to target for mutagenesis. The recent elucidation of the three-dimensional structure of *Rd* lipase determined by X-ray crystallography (12) provided necessary structural information for more precise identification of the molecular determinants of fatty acyl chain specificity of this enzyme. Utilizing computer-aided molecular modeling, we have constructed a series of single and double amino acid substitution mutants in the acyl binding groove of *Rd* lipase in an attempt to identify the molecular determinants of acyl chain length specificity. The effect of amino acid substitutions on catalytic activity and chain length specificity is discussed in relation to the structural effect of the mutation as predicted by computer-aided molecular models.

MATERIALS AND METHODS

Site-directed mutagenesis. To facilitate site-directed mutagenesis, an 1100 bp *Nco*I-*Bam*HI fragment from plasmid pET 11d-1231 (pET11d recombinant plasmid containing *Rd* prolipase open reading frame sequence; Ref. 14) was subcloned into M13BM20RF phage (Boehringer Mannheim Corp., Indianapolis, IN). Site-directed mutants were generated as detailed in the site-directed mutagenesis protocol provided by the supplier (Amersham Corp., Arlington Heights, IL). To construct double mutants, single-strand DNA containing single amino acid substitutions was used as template for an additional cycle of site-directed mutagenesis. Mutated *Nco*I-*Bam*HI fragments were sequenced (17) and cloned into plasmid pET 15b (Novagen Corp., Madison, WI) for synthesis of recombinant prolipase in *E. coli*. To obtain the same mutations in the mature form of the *Rd* lipase, a 600 bp *Kpn*I-*Eco*RI fragment encompassing the mutations in the prolipase gene was subcloned into plasmid pET 11d-f1-431 (pET 11d recombinant plasmid harboring complete *Rd* mature lipase encoding sequence; Ref. 14) in place of wild-type sequences.

Synthesis and purification of recombinant lipases. Escherichia coli BL21(DE3) cells were transformed with recombinant pET-lipase plasmids and grown in liquid culture media (M9ZB + 100 µg/mL carbenicillin) at 37°C as previously described (18). Synthesis of recombinant lipase was induced by the addition of IPTG to a final concentration of 10 µM (culture OD_{600} = 1.0). Cells were harvested 1.5 to 2 h after IPTG addition by centrifugation (5,500 × *g*, 15 min). Cell pellets were resuspended, lysed, and proteinaceous inclusion bodies obtained (11). Proteinaceous inclusion bodies were solubilized with 8 M urea and the enzyme refolded essentially as described (11) except that the refolded lipases were not further purified by affinity chromatography.

Determination of lipase activity. Initial visual screening of lipase activity utilized a rhodamine plate assay (19). BL21(DE3) cells harboring pET-prolipase plasmids were induced with IPTG and collected by centrifugation. Cells were lysed by sonicating in 50 mM Tris (pH 7.5), 2 mM EDTA, 35 µg/mL phenylmethylsulfonyl fluoride, 1.75 µg/mL leupeptin, and 0.7 µg/mL pepstatin at 4°C. Under these conditions, approximately 1 to 5% of the expressed prolipase was soluble and catalytically active (11,14). Aliquots of *E. coli* lysates containing recombinant prolipases were spotted on media containing a triacylglycerol and rhodamine B. Lipase activity was visualized under ultraviolet light.

Quantitative determination of lipase hydrolytic activity using single triacylglycerol substrates was determined titrimetrically using a VIT 90 Video Titrator (Radiometer, Copenhagen, Denmark). Standard emulsions contained 200 mM olive oil, tricaprylin or tributyrin, 15 mM $CaCl_2$, and 4.2% (wt/vol) gum arabic. Reactions were conducted at 26°C with a set point pH of 7.5. Lipase activity was calculated from the maximum rate of titrant addition using a lipase titrimetric program (20). Titrimetric assays were run in duplicate or triplicate.

Competitive hydrolysis of triacylglycerols was examined in a reaction media containing 100 mM Tris (pH 7.5), 15 mM $CaCl_2$, and equiweight mixtures of tributyrin, tricaprylin, and triolein (2.0% wt/vol). Triacylglycerol mixtures were sonicated and reactions initiated by the addition of lipase (5–10 µg/mL reaction volume). After 30 min (28°C, 250 rpm), reactions were quenched on ice and 5 mg stearyl alcohol (in 100% ethanol) added as an internal standard. Reaction mixtures were extracted with 5 vol hexane for 2 h (250 rpm). Five to 10 µL of the hexane phase were injected into a Hewlett-Packard (Avondale, PA) high-performance liquid chromatograph model 1050 ternary gradient system equipped with an Alltech-Varex Mark III evaporative light-scattering detector (Deerfield, IL) and a 3 × 100 mm LiChrosorb DIOL column (5 µm inner diameter; Chrompack, Inc., Raritan, NJ). The solvents were: A, hexane/acetic acid (1000:1, vol/vol); and B, hexane/isopropanol (100:1, vol/vol). The linear gradient timetable was: 0 min, 100:0; 8 min, 100:0; 10 min, 75:25; 40 min, 75:25; 41 min, 100:0; 60 min 100:0 (% solvent A/% solvent B). Peaks in the chromatograms were identified by comparing retention times to those of triacylglycerol standards. Standard curves were generated for tributyrin, tricaprylin, and triolein based on the injection of seven different amounts of a standard mixture of triacylglycerols. Lipolytic activities were determined by quantifying the disappearance of triacylglycerol substrates compared to control reactions with boiled lipase.

Molecular modeling. All molecular modeling work was conducted using the SYBYL (version 6.0) software package (Tripos Associates, St. Louis, MO) on a SGI Indigo2 workstation (Silicon Graphics, Mountain View, CA). Tripos forcefield parameters were used for all molecular mechanics and molecular dynamics simulations. Nonbonded interaction cutoff of 8 Å was used as was a distance-dependent dielectric constant for electrostatic interactions. All essential protein hydrogens were included in the molecular models. Kollman

United-atoms charges were assigned to all protein atoms while Gasteiger-Huckel charges were assigned to all nonprotein (e.g., lipid) atoms. Energy minimizations were performed with the Powell algorithm, using a change in the rms gradient of less than 0.05 kcal/mol/Å as the convergence criterion. The coordinates of *Rd* lipase were obtained from Z. Derewenda (University of Virginia, Charlottesville, VA). The crystal structure of *Rd* lipase consists of two molecules of the enzyme: one in the closed lid conformation and the second in a conformation intermediate between the closed and open state (12). The coordinates of the enzyme molecule with the partially open lid were used for molecular modeling and substrate docking. The lid was rearranged to a completely open conformation by using the interatomic distances between the α carbons of the lid region of *R. miehei* (Brookhaven Protein DataBase, file 4TGL) (15,16) as constraints in the *Rd* lipase in conjunction with force constants of 50 kcal/mol/Å^2. The structure was subsequently refined *via* energy minimization and molecular dynamics simulations carried out in vacuum at 50 K (total simulation time of 10 ps) followed by a final energy minimization.

To obtain molecular models of mutant *Rd* lipases, the selected amino acid residues were replaced with mutant residues. The optimal conformations of the side chains of mutated residues were obtained by performing systematic grid searches of the relevant torsional angles (at 30° increments). The relevant torsion angles explored included C_α-C_β bonds, C_β-C_α bonds (etc.) as appropriate for the particular substituted amino acid side chain. The lowest energy conformations found in these searches were then refined *via* energy minimization and molecular dynamics simulations. Molecular dynamics simulations were carried out in vacuum at 50 K (total simulation time of 10 ps) followed by a final energy minimization. These structures were utilized as initial enzyme models for docking triacylglycerol substrates.

Models of the triacylglycerol substrates were constructed and energy minimized with the SYBYL version 6.0 software. Initial models of lipase–substrate transition states were obtained by manually positioning the *sn*-3 acyl side chain in the acyl binding groove as defined by transition state analogs (15,21). To build a tetrahedral covalent lipase–substrate complex, the carbonyl carbon of the substrate was converted from its sp^2 configuration to an sp^3-hybridized carbon atom. The conversion of the carbonyl carbon to an sp^3-hybridized carbon atom was necessary to create the correct tetrahedral geometry present in the acyl–enzyme transition state intermediate. The position of the carbonyl carbon of the substrate was adjusted manually to permit hydrogen bonds between the protonated his257, the Oγ of the ser145 hydroxyl group and the oxygen leaving group of the substrate. Furthermore, the distances between the oxyanion of the transition state and the hydroxyl group and main chain amide of thr83 (which comprises the oxyanion hole) were constrained. These bond distances were constrained during the docking procedure and subsequent energy minimization and molecular dynamics simulations. Energy minima of the lipase–substrate complex were found by molecular dynamics simulations in vacuum at 50 K (total simulation time of 10 ps). Molecular dynamics simulations were conducted at low temperature (50 K) to prevent corruption of the conformation of the peptide backbone. Following molecular dynamics simulation, the conformations were subjected to a final energy minimization.

Miscellaneous. Protein concentrations were determined using the bicinchoninic acid protein assay method (Pierce Laboratories, Rockford, IL) using bovine-γ-globulin as protein standard. SDS–12.5% polyacrylamide gel electrophoresis (PAGE) was performed and protein bands on gels were visualized by silver staining (22). Estimates of the relative intensities of protein bands were obtained by video densitometry and analysis of the digitized profile with ImageQuaNT 4.2 software (Molecular Dynamics, Sunnyvale, CA).

RESULTS AND DISCUSSION

Joerger and Haas (14) initiated efforts to alter the acyl chain length specificity of *Rd* lipase through site-directed mutagenesis. Lacking a crystal structure of *Rd* lipase, conserved amino acid residues shown to form the acyl binding groove of *R. miehei* were targeted for mutagenesis in *Rd* lipase. In the present study, we extended the previous mutagenic study aided by the recent elucidation of the crystal structure of *Rd* lipase (Fig. 1). Single and double amino acid substitutions were engineered at discrete positions in the acyl binding groove based largely on structural predictions resulting from molecular dynamics simulations (Fig. 2). Several strategies for altering *Rd* lipase chain length specificity through rational mutagenesis were examined including: introduction of bulky hydrophobic trp residues at discrete positions in the acyl binding groove; creating salt bridges (ionic pairs) across the acyl binding groove by substituting polar or charged residues in the acyl binding groove; and substituting polar amino acids for hydrophobic residues in the acyl binding groove. An additional strategy involved combining selected point mutations of Joerger and Haas (14) into one mutant lipase in an attempt to develop an enzyme with greater substrate specificity.

To engineer a lipase with specificity for short-chain fatty acids, the double mutation F112W + V209W was constructed. Joerger and Haas (14) determined that substitution of trp for phe112 or val209 increased specificity for short-chain (C_4) compared to long-chain fatty acids ($C_{18:1}$). Molecular dynamics simulations of the double mutant V209W + F112W predicted that trp side chains at amino acid positions 112 and 209 of *Rd* lipase would project across the acyl binding groove, thereby posing geometric or steric constraints for the binding of fatty acids longer than butyrate in the acyl groove (Fig. 2A). When screened on rhodamine media containing tributyrin (TB), tricaprylin (TC), trilaurin (TL), or olive oil, hydrolytic activity of the V209W + F112W prolipase was visible only for media containing TB (data not shown). To quantify hydrolytic activity for prolipase and mature lipase mutants, titrimetric assays utilizing single triacylglycerol emulsions were conducted (Figs. 3 and 4). The V209W + F112W mutations resulted in

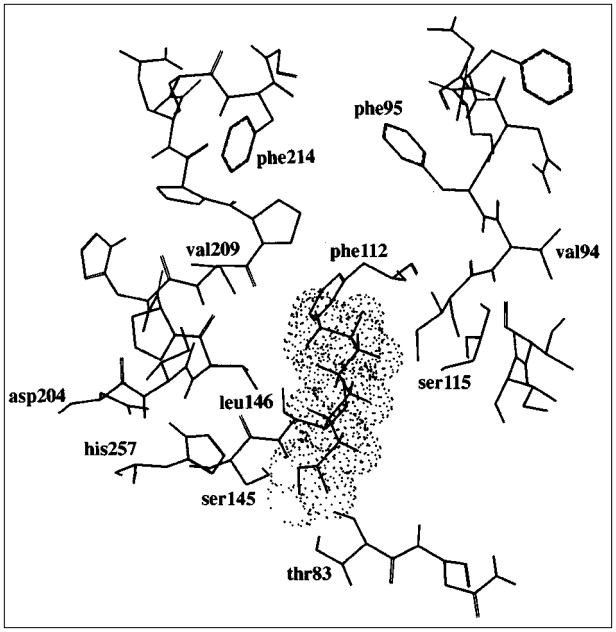

FIG. 1. Structure of the active site region of *Rhizopus delemar* lipase with the *sn*-3 acyl side chain of tricaprylin shown docked into the catalytic center. Only the *sn*-3 acyl side chain of tricaprylin is shown while the remainder of the triacylglyceride is not displayed. The catalytic residues ser, asp, and his are labeled along with thr83 which constitute the oxyanion hole. Selected residues lining the acyl binding and catalytic sites are shown. Sides chains of residues that form part of the acyl binding site targeted for mutagenesis are labeled. The displayed portion of the substrate is depicted with *van der Waals* dots. Models of the docked substrate were calculated by energy minimization and molecular dynamics simulations with bond distance constraints as defined in the Materials and Methods section.

increased specificity for TB though the specific activity of the prolipase and mature lipases for TB was reduced 10- and 1.6-fold, respectively, compared to wild-type enzyme. However, the relative activity of the V209W + F112W mutant toward TB increased substantially since hydrolysis of TC and olive oil was minimal. Relative to wild-type prolipase, the V209W + F112W showed a 9-fold increase in the ratio of TB to olive oil hydrolysis and a 15-fold increase in the ratio of TB to TC hydrolysis. Similarly, the mature lipase V209W + F112W mutant showed an 8.5- and 24-fold increase in the ratio of TB to olive oil and TB to TC hydrolysis, respectively, compared to wild-type lipase.

Quantifying the hydrolytic activities of lipases against single triacylglycerol emulsions provided an initial view of substrate specificity. A more accurate assessment of substrate specificity is obtained by competitive hydrolysis assays utilizing mixed triacylglycerol substrates. In competitive hydrolysis assay conditions, the V209W + F112W prolipase and mature lipase mutants showed strong selectivity for TB. The hydrolytic activity of prolipase V209W + F112W for TB was 10-fold greater than that observed for TC or triolein (TO) (Fig. 5). By comparison, the wild-type prolipase enzyme hydrolyzed TB, TC, and TO to a similar extent in mixed substrate assays. Mature lipase mutant V209W + F112W showed an 80-fold increase in the ratio of TB to TC hydrolysis compared to wild-type lipase while hydrolysis of TO by the V209W + F112W mature lipase was not detected under the present competitive hydrolysis conditions (Fig. 6). The results obtained with either single or mixed triacylglycerol substrates showed that the V209W + F112W double mutant exhibited much stronger selectivity for tributyrin than either of the single amino acid substitutions described by Joerger and Haas (14). The experimental results are in agreement with predictions from molecular dynamics simulations of this double mutant which suggested that the binding of medium- or long-chain length fatty acids may be sterically or geometrically constrained, thereby resulting in a mutant lipase with short-chain fatty acid specificity. It should be noted, however, that the molecular dynamics simulations could not account for the observed reduction in the specific activity of the mutant for tributyrin compared with wild-type enzyme. These observations suggest that the V209W and F112W mutations may have pleiotropic effects on protein structure that remain to be elucidated.

In an attempt to improve the specificity of *Rd* lipase for medium-chain length fatty acids, trp was substituted for V94 in the acyl binding groove. Molecular dynamic simulations predicted that this substitution may pose steric or geometric constraints to the docking of fatty acids longer than caprylate (C_8) in the acyl binding groove (Fig. 2B). The V94W mutation had a modest effect on fatty acid chain length specificity in both the prolipase and mature enzyme. In single substrate hydrolysis assays, a modest 1.6- to 1.8-fold increase in TC hydrolysis relative to TB was observed for V94W enzymes (Figs. 3 and 4). In contrast, the hydrolysis of TC relative to TO by the prolipase and mature enzyme was not altered by the V94W mutation. The results obtained with competitive hydrolysis assays utilizing mixed triacylglycerol substrates paralleled those obtained with single substrate emulsions (Figs. 5 and 6). Compared to wild-type prolipase, the V94W mutant showed a modest 30% reduction in the hydrolysis of TB and TO while the hydrolysis of TC was similar to wild-type levels. The only notable change in hydrolytic activity by introducing this mutation into mature form of *Rd* lipase was a 40% increase in hydrolysis of TC relative to TO. These results indicate that the single substitution of a bulky trp residue at the given location in the acyl binding groove did not markedly alter acyl chain length specificity as predicted by molecular dynamics simulations. The modest increase in specificity for tricaprylin, however, does suggest that a minor molecular determinant of chain length specificity may reside in this position of the acyl binding groove. Hence, it may be feasible to engineer an enzyme with much greater specificity for medium chain length fatty acids by combining the V94W mutation with mutations in other regions of the acyl binding groove shown to impact substrate specificity.

An approach to modify lipase substrate specificity is to alter the structure of the acyl binding groove by creating a salt bridge at discrete positions in the groove. The creation of a salt bridge may alter the conformation of the acyl groove,

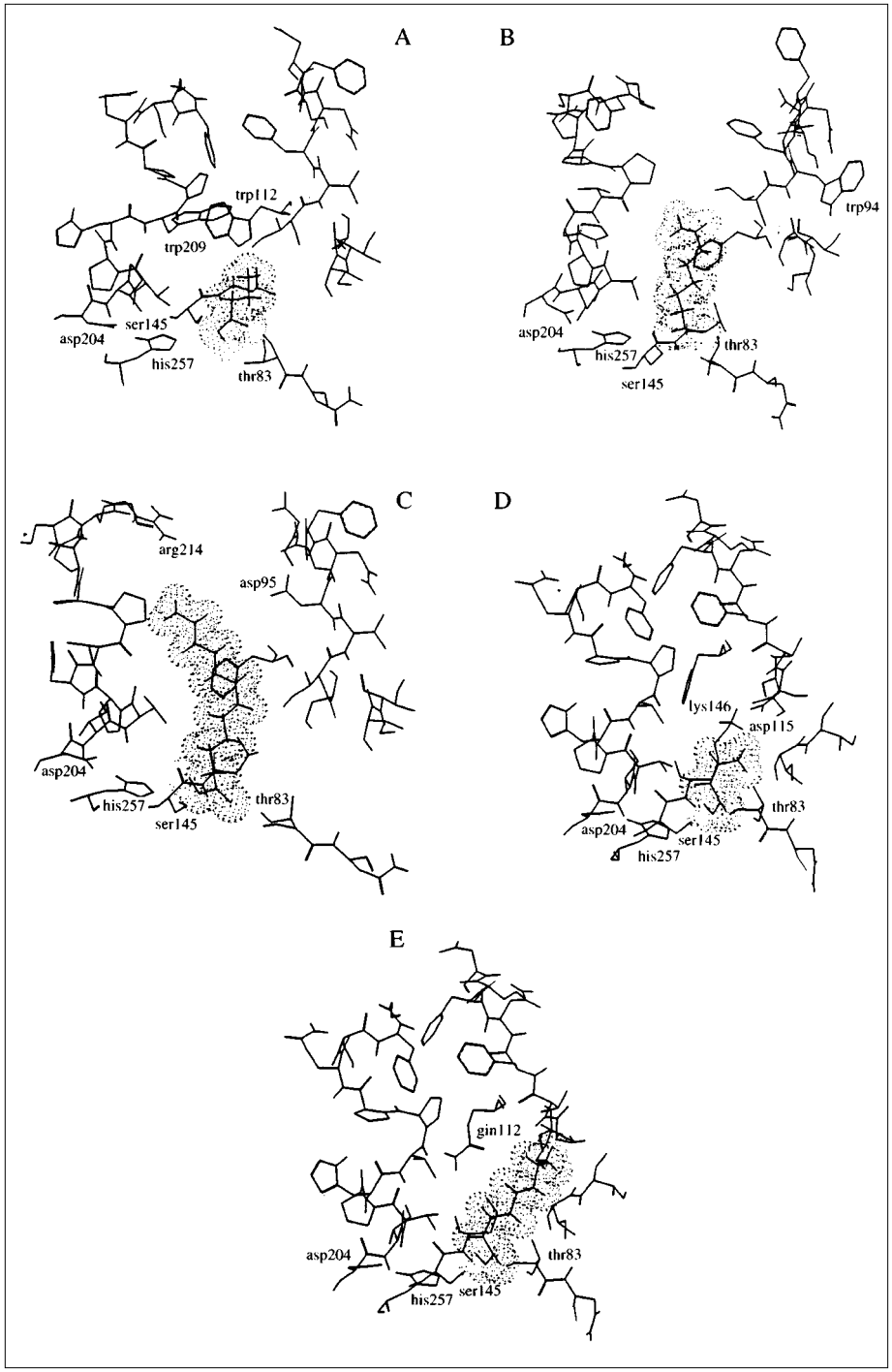

FIG. 2. Models of the active site region of mutated *Rhizopus delemar* lipase with the *sn*-3 acyl side chain of triacylglycerol substrates shown docked in the catalytic center. Only the *sn*-3 acyl side chain of each triacylglycerol is shown while the remainder of the lipid is not displayed. (A) F112W + V209W mutant with docked tributyrin (TB); (B) V94W mutant with docked tricaprylin (TC); (C) F95D + F214R with docked trilaurin (TL); (D) S115D + L146K mutant with docked TB; and (E) F112Q with docked TC. Models of the docked substrates were calculated by energy minimization and molecular dynamics simulations.

thereby constraining or enhancing the binding of select fatty acids. In an attempt to increase selectivity for medium chain length fatty acids, the double mutant F214R + F95D was engineered to position a salt bridge across the distal end of the acyl binding groove (Fig. 2C). In single substrate assays, the prolipase F214R + F95D mutant showed only a slight increase in preference for TC compared to wild-type prolipase (Fig. 3). By comparison, the mature lipase F214R + F95D

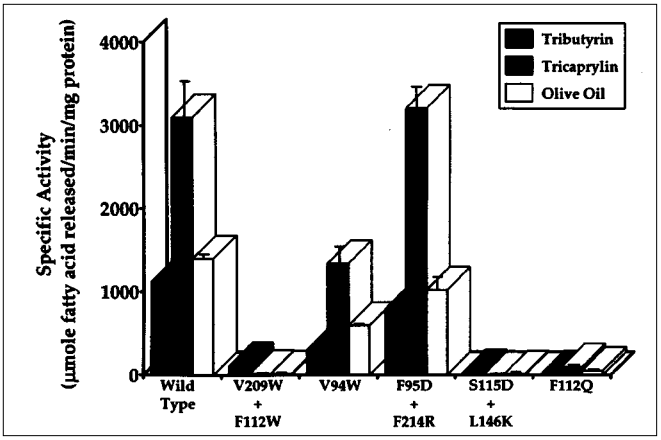

FIG. 3. Comparison between the specific activities of mutant and wild-type *Rhizopus delemar* prolipase using single triacylglycerol substrates. Specific activities were measured titrimetrically against single triacylglycerol emulsions of tributyrin, tricaprylin, or olive oil. Each specific activity is the mean of two or three independent assays.

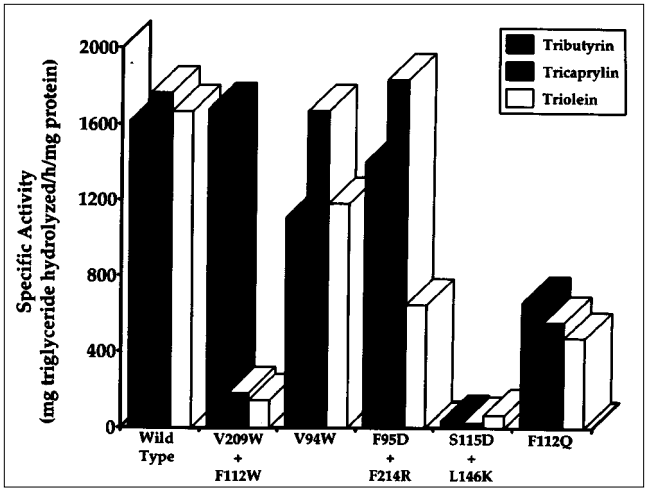

FIG. 5. Comparison between the specific activities of mutant and wild-type *Rhizopus delemar* prolipase using mixed triacylglycerol substrates. Hydrolytic activities were determined by quantifying the amounts of unhydrolyzed triacylglycerols remaining after prolipase incubation with an equiweight mixture of tributyrin, tricaprylin, and triolein. The amount of each triacylglycerol remaining after 30-min incubation with prolipase was determined by HPLC. Competitive hydrolysis assays were conducted a minimum of four times for each enzyme and a representative data set is shown.

mutation showed a 3.3-fold increase in the specific activity toward TC compared to wild-type lipase (Fig. 4). The increased specific activity of the F214R + F95D mature lipase for TC resulted in a 2-fold increase in the relative activity toward TC compared with either TB or olive oil. In competitive hydrolysis assays with triacylglycerol mixtures, the prolipase F214R + F95D mutant exhibited a 2.5-fold increase in the hydrolysis of TC relative to TO while the same mutations in the mature lipase showed a 3.1-fold increase in hydrolysis of TC relative to TO (Figs. 5 and 6). Hence, the introduction of the F95D and F214R mutations increased lipase specificity for medium chain length fatty acids especially for mutations in the mature form of *Rd* lipase. Structurally, whether a salt bridge was actually created between arg214 and asp95 has not been determined although molecular dynamics simulations predicted the ionic pairing of these residues. Nevertheless, it is evident that molecular determinants of acyl chain length specificity reside in this region of the acyl binding site and are amenable to modification.

The double mutation S115D + L146K was engineered to determine the feasibility of introducing charged residues nearer the catalytic center with an objective of impacting acyl chain length specificity (Fig. 2D). Molecular dynamics simulations predicted that the S115D + L146K mutations would create a salt bridge across the acyl binding groove approximately 8 Å from the catalytic center. The position of the salt bridge was predicted to hinder the docking of medium- and

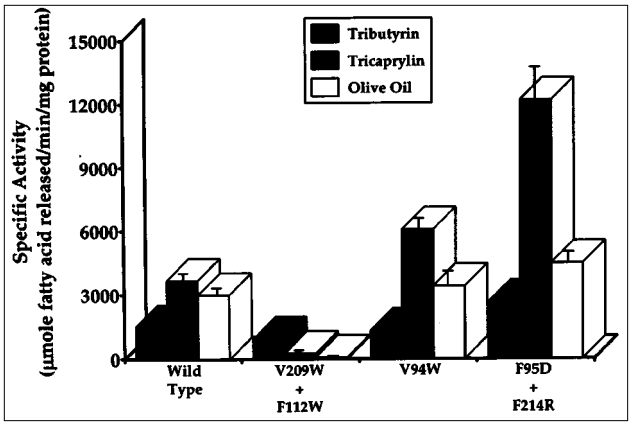

FIG. 4. Comparison between the specific activities of mutant and wild-type mature *Rhizopus delemar* lipase using single triacylglycerol substrates. Specific activities were measured by titrimetric assay against single triacylglycerol emulsions as described for Figure 3.

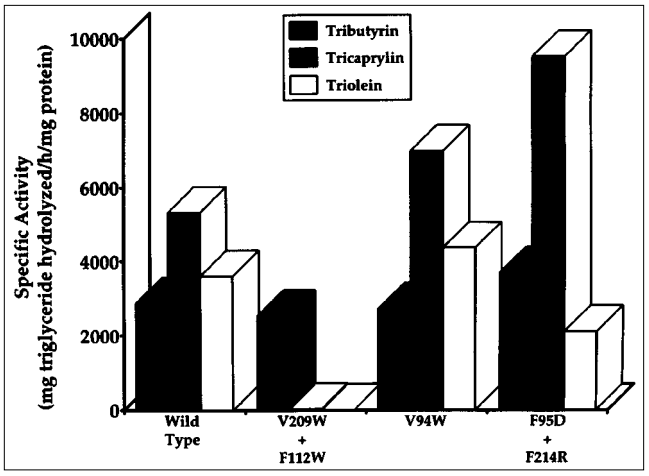

FIG. 6. Comparison between the specific activities of mutant and wild-type *Rhizopus delemar* mature lipase using mixed triacylglycerol substrates. Specific activities of mutant and wild-type mature lipases were determined as described for Figure 5.

long-chain fatty acids and favor the docking of short (C_4) fatty acids in the acyl binding groove. An additional mutation, F112Q, was engineered to determine whether the substitution of a polar residue near the rim of the acyl binding groove would impact acyl chain length specificity (Fig. 2E). The residue phe112 was targeted for mutagenesis since it had been determined that this site is amenable to substitution and molecular dynamics simulations predicted that the docking of long fatty acid chains in the acyl binding groove would be sterically hindered by a polar residue at this position. Hydrolysis assays revealed that the prolipase S115D + L146K mutant was catalytically inactive (Fig. 3). Similarly, introduction of a polar gln for phe112 largely eliminated activity in single substrate emulsions (Fig. 3) and reduced the hydrolytic activity for all three triacylglycerols in mixed emulsions assays (Fig. 5). To determine whether the loss of catalytic activity of the S115D + L146K mutant is due to one or both amino acid substitutions, the single amino acid mutations were examined for catalytic activity. Rhodamine plate and single substrate hydrolysis assays showed that neither the S115D nor the L146K mutants possessed catalytic activity (data not shown). The close proximity of S115 and L146 to the catalytic triad of ser145, his257 and asp204 and the oxyanion hole (thr83) may explain the disruption of catalytic activity. L146 resides within 4 Å of S145 (distance between α carbons). In contrast, F112 is located approximately 12 Å from catalytic ser145 and is located at the rim of the acyl binding groove. Nevertheless, these amino acid substitutions largely eliminated the catalytic activity of the prolipase. The loss of catalytic activity may relate to the disruption of some step in catalysis after the binding of the substrate or to the prevention of efficient penetration of the lipid interface, resulting in lower activity (23). It should be noted that the introduction of polar or charged residues in the acyl binding groove is not necessarily lethal as evidenced by the F95D + F214R mutant and a second double mutant, V206T + F95D, which show strong substrate specificity for medium chain length fatty acids (Klein, R.R., Villeneuve, P., King, G., McNeill, G.P., Moreau, R.A., and Haas, M.J. manuscript in preparation). Hence, our present knowledge of lipase structure–function relationships does not permit an accurate prediction of the lipase phenotype that results from the introduction of charged or polar residues in the acyl binding groove.

In summary, the present study describes attempts toward engineering novel biocatalysts *via* protein engineering of *Rd* lipase. Similar approaches for the rational modification of this lipase are being conducted in other laboratories as well (24). The observed mutant phenotypes indicate that tailoring the substrate specificity of *Rd* lipase is feasible as evidenced by the F112W + V209W and F95D + F214R double mutants. As expected, the major difficulty was predicting the effect of the mutation on catalytic activity. Increasing the likelihood of creating the desired catalytic activity may require mutagenic strategies that simultaneously introduce multiple mutations in a gene (25–27). Computer-aided molecular models may make the process more efficient by identifying critical regions (cassettes) to target for multiple amino acid substitutions, insertions, or deletions. The *Rd* prolipase expression system is well suited for such a mutagenic strategy since a rapid fluorescence visual screen for hydrolytic activity has been developed (14). Unfortunately, the prolipase crystal structure has not been solved, and the presence of the propeptide has significant impact on substrate specificity. Resolution of the crystal structure of the *Rd* prolipase would aid in our understanding of protein structure–function relationship and aid in the tailoring of lipase substrate specificity through protein engineering.

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Peroxidase-Catalyzed Oxidation of β-Carotene in HL-60 Cells and in Model Systems: Involvement of Phenoxyl Radicals

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ABSTRACT: Recent studies provide extensive evidence for the importance of carotenoids in protecting against oxidative stress associated with a number of diseases. In particular, reactions of carotenoids with phenoxyl radicals generated by peroxidase-catalyzed one-electron metabolism of phenolic compounds may represent an important antioxidant function of carotenoids. To further our understanding of the antioxidant mechanisms of carotenoids, we used in the present work two different phenolic compounds, phenol and a polar homologue of vitamin E (2,2,5,7,8-pentamethyl-6-hydroxychromane, PMC), as representatives of two different types of phenols to study reactions of their respective phenoxyl radicals with carotenoids in cells and in model systems. We found that phenoxyl radicals of PMC did not oxidize β-carotene in either HL-60 cells or in model systems with horseradish peroxidase (HRP)/H_2O_2. In contrast, the phenoxyl radicals generated from phenol (by native myeloperoxidase in HL-60 cells or HRP/H_2O_2 in model systems) effectively oxidized β-carotene and other carotenoids (canthaxanthin, lutein, lycopene). One-electron reduction of the phenoxyl radical by ascorbate (assayed by electron spin resonance-detectable formation of semidehydroascorbyl radicals) prevented HRP/H_2O_2-induced oxidation of β-carotene. PMC, but not phenol, protected β-carotene against oxidation induced by a lipid-soluble azo-initiator of peroxyl radicals. No adducts of peroxidase/phenol/H_2O_2-induced β-carotene oxidation intermediates with phenol were detected by high-performance liquid chromatography–mass spectrometry analysis of the reaction mixture. Since carotenoids are essential constituents of the antioxidant defenses in cells and biological fluids, their depletion through the reaction with phenoxyl radicals formed from endogenous, nutritional and environmental phenolics, as well as phenolic drugs, may be an important factor in the development of oxidative stress.
Lipids 32, 131–142 (1997).

There are three major paths by which one-electron free radical intermediates of oxidation of phenolic compounds, phenoxyl radicals, are generated in cells and biological fluids. One is associated with antioxidant defense mechanisms and occurs by the interaction of natural and/or synthetic phenolic chain breaking antioxidants (e.g., vitamin E, ubiquinol, probucol) with peroxyl radicals (1–4):

$$ROO + Ph\text{-}OH \rightarrow ROOH + Ph\text{-}O$$

Another involves the one-electron catalytic oxidation of low molecular weight phenols by several important enzymes (e.g., peroxidases, tyrosinase, prostaglandin synthase) (5–8). In the third path, tyrosyl residues in proteins are converted to phenoxyl radicals, and are catalytic intermediates detectable in a number of proteins and enzymes such as ribonucleotide reductase (9). While in the latter case, the reactivity of the tyrosine phenoxyl radical (Tyr122 in the B_2 subunit) is essential for oxidation of two thiol groups (Cys754 and Cys759 in the B_1 subunit), and the formation of the thiyl radical and its oxidative attack on ribose (9,10), the relative reactivities of the various low molecular weight phenoxyl radicals formed in the first two paths may also be very important physiologically. The different reactivities of the phenoxyl radicals formed from phenolic free radical scavengers determine their relative antioxidant effectiveness (vs. their prooxidant chain-propagating effects). Formation of phenoxyl radicals from toxic phenolic compounds may represent the key mechanism by which they exert their cytotoxic and mutagenic effects, causing futile "thiol pumping" and yielding oxygen-centered free radicals (8). Thus, the relative reactivities of phenoxyl radicals may be pivotal for both normal metabolism and pathological mechanisms.

Recent studies demonstrate that reactivities of phenoxyl radicals can be surprisingly high. For example, experiments in model systems (nonaqueous solvents) have shown that rate constants for hydrogen atom abstraction by the phenoxyl radicals are 100–300 times greater than those for peroxyl radicals (11). Several studies also demonstrate that both unhindered and hindered phenoxyl radicals can directly oxidize thiols (glutathione, dihydrolipoic acid) and protein sulfhydryls (8,12,13). Enzymes such as peroxidases (e.g., myeloperoxidase) and prostaglandin synthase, as well as the peroxidase activity of hemoglobin and myoglobin, are promoters of low density lipoprotein (LDL) lipid oxidation

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Abbreviations: AAPH, 2,2'-azo-*bis*(2-amidinopropane)dihydrochloride; AMVN, 2,2'-azo-*bis*(2,4-dimethylvaleronitrile); DOPC, 1,2-dioleoylphosphatidylcholine; ESR, electron spin resonance; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; LDL, low density lipoprotein; MS, mass spectrometry; PMC, 2,2,5,7,8-pentamethyl-6-hydroxychromane; PMSF, phenylmethylsulfonyl fluoride; THF, tetrahydrofuran; UV, ultraviolet.

(14–16). It has been suggested that the mechanism of thiol oxidation involves the generation of an intermediate radical, such as the phenoxyl radical of tyrosine or vitamin E, which then rapidly consumes antioxidants and propagates free radical-based lipid peroxidation (17). Direct reactions of phenoxyl radicals with lipids or lipid antioxidants (e.g., carotenoids), however, have not been studied in cells or under physiologically relevant conditions.

The biological activity of carotenoids is a direct consequence of the chemistry of their long polyene chain: a highly reactive, electron-rich system of conjugated double bonds susceptible to attack by electrophilic reagents, thus responsible for the ability of carotenoids to act as reductants (and their susceptibility to oxidation). Carotenoids react rapidly with peroxyl radicals and can compete with the allylic hydrogens of polyunsaturated fatty acids in membranes, thereby acting as antioxidants (18–23). The antioxidant effect of β-carotene is apparent at low O_2 concentrations in which the carotenyl radical functions as an efficient chain terminator. In the presence of O_2, however, the β-carotene peroxyl radical is formed, which triggers further oxidations (24). While reactions of carotenoids with peroxyl radicals as well as with oxygen radicals and singlet oxygen have been extensively studied (18,19,22,25–27), their interactions with phenoxyl radicals have not been investigated. The interrelationships between carotenoids and phenoxyl radicals of phenolic antioxidants and cytotoxic phenols are of special importance in view of their potential role in attenuating or enhancing oxidative stress.

In the present work, we used two different phenolic compounds, phenol and 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) (a polar homologue of vitamin E), as representatives of two different types of low molecular weight phenols. The former, a cytotoxic compound, and the latter, an antioxidant protector, were used to study reactions of their respective phenoxyl radicals with carotenoids in cells and in model systems. We present evidence that the phenoxyl radical of PMC does not oxidize β-carotene in HL-60 cells nor in model systems. In contrast, the phenoxyl radical generated from phenol (by native myeloperoxidase in HL-60 cells or horseradish peroxidase (HRP) in model systems) effectively oxidizes β-carotene and other carotenoids. The oxidation of phenol was prevented by ascorbate, which reduces phenoxyl radicals to form the semidehydroascorbyl radical.

MATERIALS AND METHODS

Reagents. Acetonitrile, toluene, and acetic acid were obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of liposomes and "micelles." Solutions of 1,2,-dioleoylphosphatidylcholine (DOPC) in chloroform and β-carotene in tetrahydrofuran (THF) were mixed and the solvents evaporated with a stream of N_2. After evaporation, phosphate buffer (50 mM) containing NaCl (100 mM), pH 7.4, was added and the suspension was sonicated (six 15-s pulses in an ice bath) using a tip sonicator (Ultrasonic Homogenizer 4710 Series; Cole-Parmer Instrument Co., Chicago, IL). The concentrations of β-carotene and DOPC were 20 and 510 μM, respectively. For our experiments, solutions of 2×10^{-3} M β-carotene were prepared in THF, 10 μL of which was injected into 1 mL of phosphate buffer. Spectrophotometric analysis showed a final concentration of 20 μM β-carotene in the 1% THF solution. The structure of the β-carotene/THF complex in aqueous solution is unknown. It is known that THF in water forms azeotropic solutions, so the likely structure is a cage of THF around one or more β-carotene molecules, with the latter maintained in a constrained, fluid state. Presumably, the oxygen atom of THF is directed outward toward the aqueous environment, while its nonpolar end solubilizes the carotenoid. Use of THF as a co-solvent allows the highly lipophilic carotenoids to be solubilized in an aqueous medium, and in a readily bioavailable form (which, for convenience, we term as "micelles"). Except for lycopene, which was not homogeneously dispersed in the phosphate buffer and was better analyzed by integration into DOPC liposomes, all other carotenoids were prepared by the same method used for β-carotene.

HL-60 cell culture. HL-60 cells (obtained from Prof. J.C. Yalowich, Department of Pharmacology, University of Pittsburgh) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere. Cells from passages 25–40 were used in experiments. The density of cells at collection time was 1.5×10^6 cells/mL. Cell viability was determined microscopically by trypan blue exclusion.

Myeloperoxidase activity. Myeloperoxidase activity in HL-60 cells was determined by the oxidation of guaiacol (28). Cells were disrupted by lysis with Tris HCl (100 mM), NaCl (200 mM), EDTA (5 mM), and phonylmethylsulfonyl fluoride (PMSF) (0.1 mM), pH 7.4. Aliquots of the lysate were combined with 3-amino-1,2,4-triazole (2.5 mM), cetyltrimethylammonium bromide (0.03%), and guaiacol (15 mM) in phosphate buffer (100 mM), pH 7.4. Myeloperoxidase activity was determined by the change in absorbance at 470 nm using $\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$. The enzyme activity was determined to be 5.4 nmol/min/10^6 cells at 20°C.

Incorporation of β-carotene into HL-60 cells. HL-60 cells were diluted to a density of 1.5×10^6 cells/mL in L1210 buffer containing NaCl (115 mM), KCl (5 mM), $MgCl_2$ (1 mM), NaH_2PO_4 (5 mM), glucose (10 mM) and Hepes (25 mM), pH 7.4. β-Carotene was dissolved in THF, then added to the cell suspension to give a final concentration of 50 μM. The β-carotene-enriched cells were incubated at 37°C in a shaking bath in sterile lab air in the dark for 30 min. After incubation, β-carotene not associated with the cells was removed by centrifugation at $800 \times g$ for 10 min. The cells collected after this procedure were determined to be 96% viable.

Incubation of HL-60 cells containing β-carotene with phenol and PMC. β-Carotene-enriched cells were diluted to a density of 1.0×10^6 cells/mL in L1210 buffer. Phenol (300 μM)

or PMC (300 μM) were added to the cell suspension and incubated in the presence or absence of H_2O_2 (400 μM) at 37°C. Reactions were terminated by the addition of sodium azide (4 mM). The cells were determined to be 95% viable following this procedure.

High-performance liquid chromatography (HPLC) assay of β-carotene. Aliquots (200 μL) were taken from cell suspensions at given time intervals and transferred to borosilicate glass tubes containing chloroform/methanol (2:1, vol/vol; 1 mL) then vortexed for 1 min. This mixture was centrifuged for 5 min at 1000 × *g*, and the upper aqueous phase was discarded. The lower organic layer was evaporated to dryness under N_2 and the residue was reconstituted in methanol/toluene (3:1, vol/vol) for HPLC analysis. A reversed-phase C_{18} HPLC column [(ODS Hypersil, 5 μm particle size, 4.6 × 200 mm; Hewlett-Packard, Palo Alto, CA)] was employed for separations on a Shimadzu LC-10A HPLC with an LC-60 pump equipped with an SPD-10AV ultraviolet (UV) detector (Tokyo, Japan). Chromatograms were generated from effluent absorbance at 450 nm. The data acquired were exported from the SPD-10AV UV detector using Shimadzu EZChrom software. The mobile phase, used at a flow rate of 1 mL/min, consisted of methanol containing 0.5% aqueous ammonium acetate (wt/vol)/toluene (33:7, vol/vol). The retention time for β-carotene under these conditions was 8.7 min. Control experiments showed that the recovery of β-carotene with this procedure was >98%.

HPLC–mass spectrometric (MS) analyses. Pneumatically-assisted electrospray mass spectra of β-carotene and its oxidation products were obtained with a Perkin Elmer/Sciex API 1 mass spectrometer (Thornhill, Ontario, Canada) equipped with an atmospheric pressure ionization source and an articulated IonSpray interface maintained at 5 kV. The orifice was operated at 70 V, high purity N_2 flowing at 0.6 L/min and heated to 55°C served as the curtain gas, and high purity air maintained at 40 psi was used for nebulization. Samples were introduced using a Hewlett-Packard 1090 Series II liquid chromatograph equipped with a 1040A diode array detector, connected in tandem to the mass spectrometer with glass capillary tubing. Analytes in the samples were separated with a 2.1 × 100 mm Hewlett-Packard 5 μm Hypersil ODS C_{18} column using methanol containing 0.15% aqueous ammonium acetate (wt/vol)/toluene (19:1, vol/vol) as the mobile phase, flowing at a rate of 0.2 mL/min. Mass spectra were acquired over the range *m/z* 200 to *m/z* 1500 with a resolution of *m/z* 0.1 at the rate of 9–12 s/scan.

HPLC assay of phenol. Aliquots (200 μL) were taken from the reaction mixtures at given time intervals and transferred to borosilicate glass tubes containing 200 μL of CH_3OH. The dispersion thus formed was filtered through a 1 mL C_{18} Sep-Pak cartridge (Waters Division of Millipore, Millipore Co., Milford, MA), and the filtrate was analyzed by HPLC using the 4.6 × 200 mm column and the Shimadzu LC system (detection was by absorbance at 272 nm). The mobile phase used was CH_3OH/H_2O (31:19, vol/vol) adjusted to pH 3.1 with CH_3COOH at a flow rate of 1 mL/min. Under these conditions the retention time for phenol was 4.8 min. Recovery of phenol using the above described procedure was >98%.

HPLC assay of PMC. Aliquots (200 μL) were taken at given time intervals and transferred from the reaction mixtures into borosilicate glass tubes. The PMC extraction was carried out as described (29). SDS (100 mM, 200 μL) was added to the samples, and briefly mixed by vortexing. Reagent-grade ethanol (400 μL) and hexane (400 μL) were added and the resulting mixtures were vigorously mixed by vortexing for 2 min. The mixtures were centrifuged for 5 min at 1000 × *g* to separate the layers. Each upper phase was transferred to a small borosilicate glass tube and dried under N_2. The residues were separately dissolved in ethanol and analyzed by HPLC using the 4.6 × 200 mm column and the Shimadzu LC system (detection was by absorbance at 292 nm). The mobile phase used was CH_3CN/H_2O (8:5, vol/vol) adjusted to pH 3.0 with CH_3COOH at a flow rate of 1 mL/min. Under these conditions the retention time for PMC was 8.6 min. Control experiments showed that recovery of PMC with this procedure was >98%.

Electron spin resonance (ESR) spectral detection of the PMC phenoxyl and semidehydroascorbyl radicals. ESR measurements were performed on a JEOL-RE1X spectrometer (Tokyo, Japan) at 25 and 37°C in gas-permeable Teflon tubing (0.8 mm internal diameter, 0.013 mm thickness; obtained from Alpha Wire Corp., Elizabeth, NJ). The tube (approximately 8 cm in length) was filled with 60 μL of sample, folded into quarters, and placed in an open 3.0 mm internal diameter ESR quartz tube in such a way that all of the sample was within the effective microwave irradiation area. Spectra of the PMC phenoxyl radical were recorded at 335.5 mT center field, 40 mW power, 0.2 mT field modulation, 10 mT sweep width, a receiver gain of 2000, and a 0.3-s time constant. Spectra of the semidehydroascorbyl radical were recorded at 335.5 mT center field, 10 mW power, 0.04 mT field modulation, 10 mT sweep width, a receiver gain of 1000, and a 0.3-s time constant.

Spectrophotometric assay of β-carotene oxidation in model systems. In "micelles" and liposomes, carotenoids were oxidized with HRP (0.1–1.0 U/mL)/H_2O_2 (400 μM) system at 25°C. 2,2′-Azo-*bis*(2-amidinopropane)dihydrochloride (AAPH) (50 mM)- or 2,2′-azo-*bis*(2,4-dimethylvaleronitrile) (AMVN) (1.0 mM)-induced oxidation of β-carotene in DOPC liposomes was carried out at 37°C. When HRP (0.1 U/mL) was used for oxidation of β-carotene in both aqueous solutions and in liposomes, the oxidation was linear for 300 s. When the oxidation of β-carotene was induced by more than 0.1 U/mL HRP, the reaction was linear for 120 s. The rate of β-carotene consumption, in μM/min, was determined using these time intervals. The numbers were obtained from the formula: μM/min = $(C_0\text{-}C)/t$, where C_0 was the concentration of β-carotene at time zero (20 μM), C was its measured concentration (μM) at different time points in the reaction when the oxidation was linear, and t was the time in s when the measurement was made. The rate constants were obtained with the formula: $k = (2.303/t) \times \log(C_0/C)$, where t

was the time in s at which a measurement was made (when the oxidation of β-carotene was linear), C_0 was the concentration (from the optical density) of β-carotene (20 μM) at time zero, and *C* was its concentration at the given time point in the linear portion of the oxidation reaction. In most experiments, serial UV-VIS spectra of carotenoids were recorded in the range 350–600 nm at scanning intervals of 2 min. A Shimadzu 160U spectrophotometer (equipped with Shimadzu PC 160 PLS software) was employed for all UV-VIS measurements. In some experiments, carotenoids were extracted from "micelles" or liposomes at specific time intervals with chloroform/methanol (2:1, vol/vol), solvents were evaporated under N_2, the residue was dissolved in THF, then analyzed spectrophotometrically.

RESULTS

Effects of H_2O_2 and phenolic compounds (phenol and PMC) on β-carotene in HL-60 cells. Treatment of HL-60 cells with β-carotene resulted in its incorporation to the level of 0.33 nmol/10^6 cells. Such treatment and loading levels did not produce cytotoxic effects in the time frame of our experiments. Incubation of β-carotene-loaded cells with phenol/H_2O_2 resulted in the consumption of β-carotene to 65% of its initial level during the first 5 min (Fig. 1), and a further decrease to 44% of its initial level after 15 min (data not shown). β-Carotene levels did not significantly change in control cells incubated for the same time period without phenol/H_2O_2. Similarly, when the incubation medium contained all reactants except phenol or H_2O_2, no significant decrease of the β-carotene content was observed. In cells pretreated with azide, an inhibitor of myeloperoxidase, the β-carotene-consuming effect of phenol/H_2O_2 was not observed.

In contrast to phenol, addition of PMC/H_2O_2 did not affect β-carotene content in preloaded HL-60 cells. Neither PMC alone nor the PMC/H_2O_2 combination in cells treated with azide caused any detectable decrease in β-carotene content.

H_2O_2-induced oxidation of phenol and PMC in β-carotene-loaded HL-60 cells. In the absence of H_2O_2, HL-60 cells loaded with β-carotene oxidized phenol or PMC slowly. The rate of oxidation of PMC under such conditions was three times higher than that of phenol (Table 1). In the presence of H_2O_2, the oxidation of phenol was increased about 15-fold, while that of PMC was increased by less than 3-fold: i.e., the rate of phenol oxidation was enhanced to twice that of PMC by addition of H_2O_2. Since, in the presence of H_2O_2, oxidation of phenol and PMC occurred at comparable rates, the dramatic difference in the oxidation of β-carotene caused by PMC and phenol (lack of oxidation by PMC/H_2O_2 and significant oxidation by phenol/H_2O_2) cannot be attributed simply to differences in the rates of oxidation of the two phenolic compounds.

Effect of phenol and PMC on the consumption of β-carotene induced by HRP/H_2O_2. We wished to gain a better understanding of the mechanism(s) underlying the different effects of phenol and PMC on the consumption of β-carotene in HL-60 cells. A simple model system consisting of HRP/H_2O_2 was thus employed to measure the oxidation of β-carotene and several other carotenoids.

Since β-carotene has characteristic absorbance features in the visible range, its oxidation can be studied spectrophotometrically (Fig. 2). In the absence of HRP/H_2O_2, β-carotene was stable, and only small changes in its absorption spectrum were observed (data not shown). HRP/H_2O_2 caused slow oxidation of β-carotene that was substantially accelerated by the addition of phenol (Fig. 2B). A simultaneous increase in absorbance at 412 nm due to accumulation of phenol oxidation products was observed (30). The consumption of β-carotene was directly proportional to the amount of HRP added (Fig. 3). When sodium azide was added to the incubation mixture containing HRP/H_2O_2, the β-carotene level remained constant regardless of the presence or absence of phenol.

In contrast to phenol, PMC did not enhance HRP/H_2O_2-

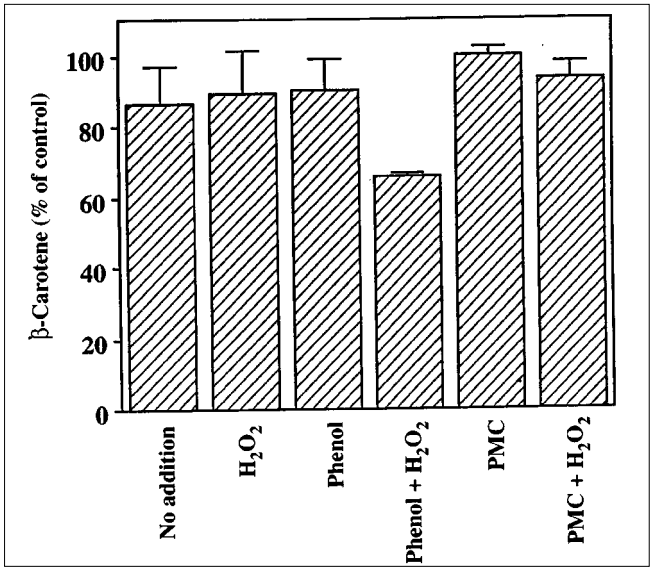


FIG. 1. Effect of phenol and 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) on the oxidation of β-carotene in HL-60 cells in the presence or absence of H_2O_2. Reaction conditions: β-carotene-loaded HL-60 cells (1.0×10^6) were incubated in L1210 medium in the presence of phenol (300 μM) or PMC (300 μM) and H_2O_2 (400 μM) at 37°C. The initial concentration of β-carotene was 0.33 nmol/10^6 cells. Results are means + standard deviations ($n = 3$).

TABLE 1
Rate of Phenol and PMC Oxidation in HL-60 Cells by Endogenous Myeloperoxidase

Additions	nmol/min/1 × 10^6 cells	
	Phenol[a]	PMC
no H_2O_2	0.21 ± 0.01	0.61 ± 0.02
H_2O_2	3.31 ± 0.10	1.61 ± 0.08

[a] The concentrations of phenol and 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) in the incubation system were 300 μM; that of H_2O_2 was 400 μM. The rates of oxidation of phenol and PMC in HL-60 cells were linear during 5 min after induction of oxidation. Data from this time period was used for determination of the oxidation rates of these compounds.

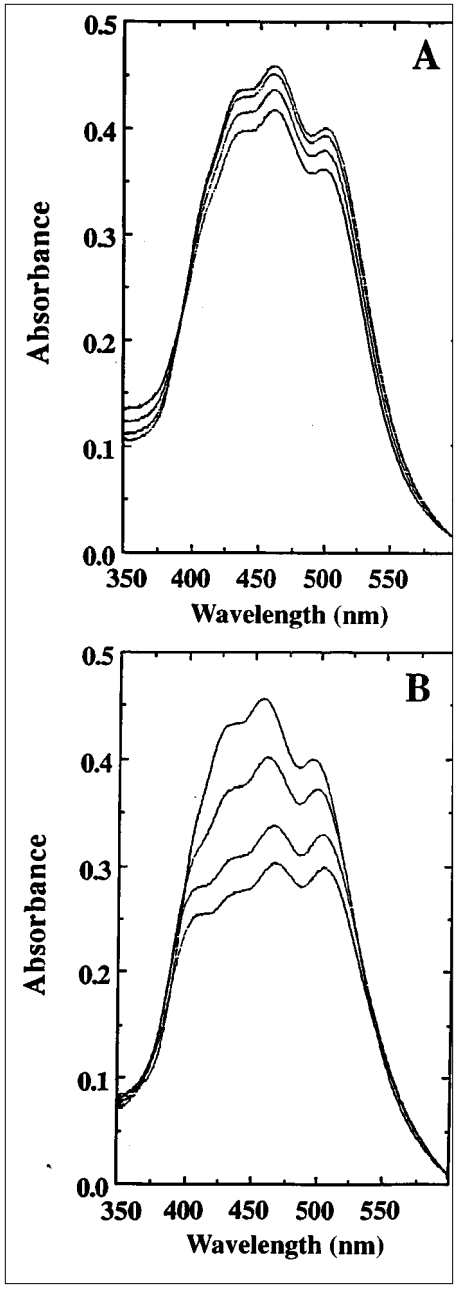


FIG. 2. Intensity decay of and changes in absorbance spectra of β-carotene "micelles" exposed to horseradish peroxidase (HRP)/H_2O_2 (A) and HRP/H_2O_2/phenol (B) at 25°C. Reaction conditions: β-carotene dissolved in tetrahydrofuran was added to phosphate buffer (50 mM), containing NaCl (100 mM), pH 7.4, to give a final concentration of 20 μM. A spectrum was taken, HRP (0.1 U/mL), H_2O_2 (400 μM), and phenol (100 μM) were then added, and spectra were recorded at 5, 15, and 30 min.

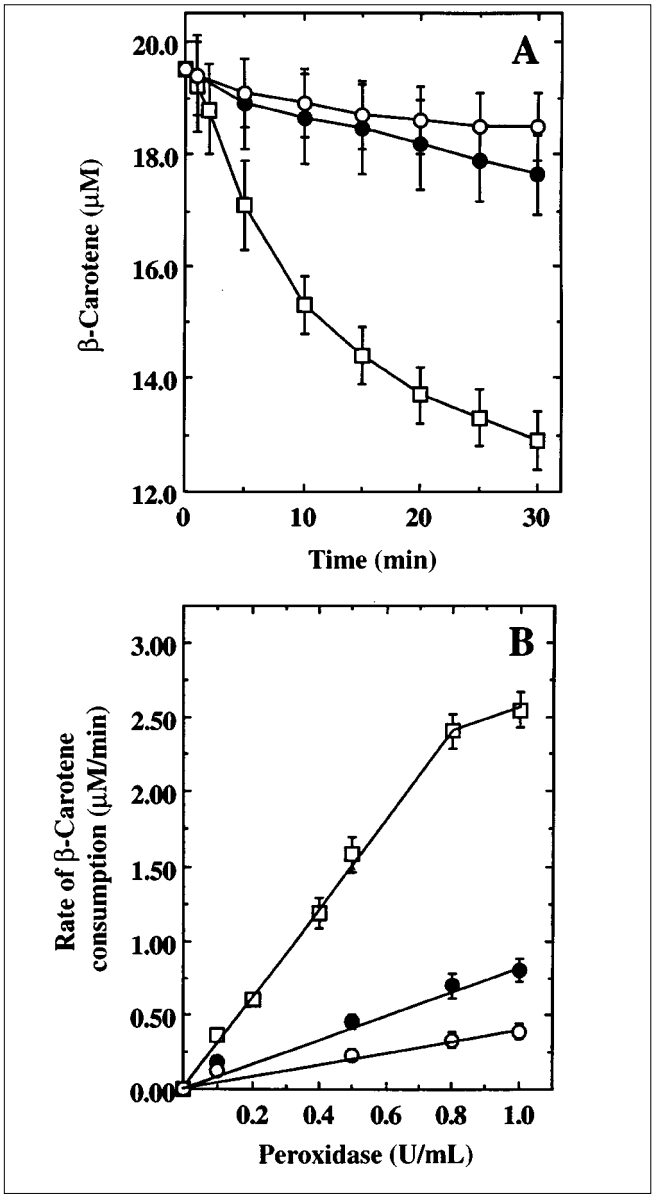


FIG. 3. Time course (A) and dependence on HRP concentration (B) of β-carotene oxidation by HRP/H_2O_2 and phenol (□), PMC (○), or no phenolic compound (●). Reaction conditions: incubation was performed in phosphate buffer (50 mM) containing NaCl (100 mM), pH 7.4, using β-carotene (20 μM), HRP (its concentration in A was 0.1 U/mL), H_2O_2 (400 μM), and phenol (100 μM) or PMC (100 μM, added as a concentrated ethanolic solution). See Figures 1 and 2 for abbreviations.

induced consumption of β-carotene. No oxidation of β-carotene occurred during incubation with the peroxidase in the absence of H_2O_2.

In order to determine which of the products of phenol oxidation (e.g., quinones, polyhydroxylated products, or intermediates such as the phenoxyl radical) were involved in β-carotene degradation in the HRP/H_2O_2/phenol system, β-carotene was added to an incubation mixture containing products of the exhaustive oxidation of phenol. Incubation of β-carotene with pre-formed (and thus likely not the phenoxyl radical) oxidation products did not induce its consumption (data not shown). Repeated addition of phenol, however, stimulated the oxidation of β-carotene. Thus, the phenoxyl radical appeared to be the sole or major contributor to the degradation of β-carotene in the HRP/H_2O_2/phenol reaction.

Analysis by HPLC–MS of the consumption of β-carotene

in the presence of phenol by HRP/H_2O_2. To determine if β-carotene-phenol adducts were formed during peroxidase-catalyzed oxidation, we examined the reaction mixtures described above by HPLC–UV-electrospray MS. As reported by Liebler and McClure, detected as a weak quasi-molecular ion of *m/z* 537.4 ([M + H]+) β-carotene 536.4 amu, and structures derived from it contained a substantial mass defect of ca. 0.5 amu (31). When the reactions were quenched by the addition of azide, a single major product appeared with an HPLC retention time identical to that of β-carotene, but an apparent mass of 549.4 amu (i.e., detected as an assumed quasi-molecular ion of *m/z* 550.4 ([M + H]+)). This product had a UV spectrum that was essentially indistinguishable from that of β-carotene, and was not formed when reactions were quenched by cooling to 0°C. Because of its odd mass and the lack of its appearance when azide was omitted, we can only tentatively identify this product as an azide adduct of β-carotene (detected in the mass spectrometer after loss of N_2), possibly arising from reaction of azidyl radical with β-carotenyl radical (32).

In the absence of azide, numerous products were detected after the cooled reaction mixtures were lyophilized, reconstituted in methanol/toluene, and promptly analyzed by HPLC–MS (Fig. 4). Two of the minor products were identified as likely arising from simple oxygenation of β-carotene, yielding quasi-molecular ions of *m/z* 551.4 ([M + (O − 2 H) + H]$^+$) and *m/z* 567.5 ([M + (O_2 − 2 H) + H]$^+$). The major products from this reaction exhibited dramatically altered (mainly blue-shifted) UV spectra and high molecular masses (>700 amu). We can only assume that these products arose from a complex set of addition and termination reactions of β-carotenyl radicals and their disproportionation products with themselves and oxygen. We found no evidence of any simple phenol adducts with β-carotene in the mass range of 625–650 amu (mono addition to β-carotene or its oxygenated adduct) nor 720–760 amu (*bis* addition to β-carotene or its oxygenated adducts).

ESR detection of semidehydroascorbyl radical and effects

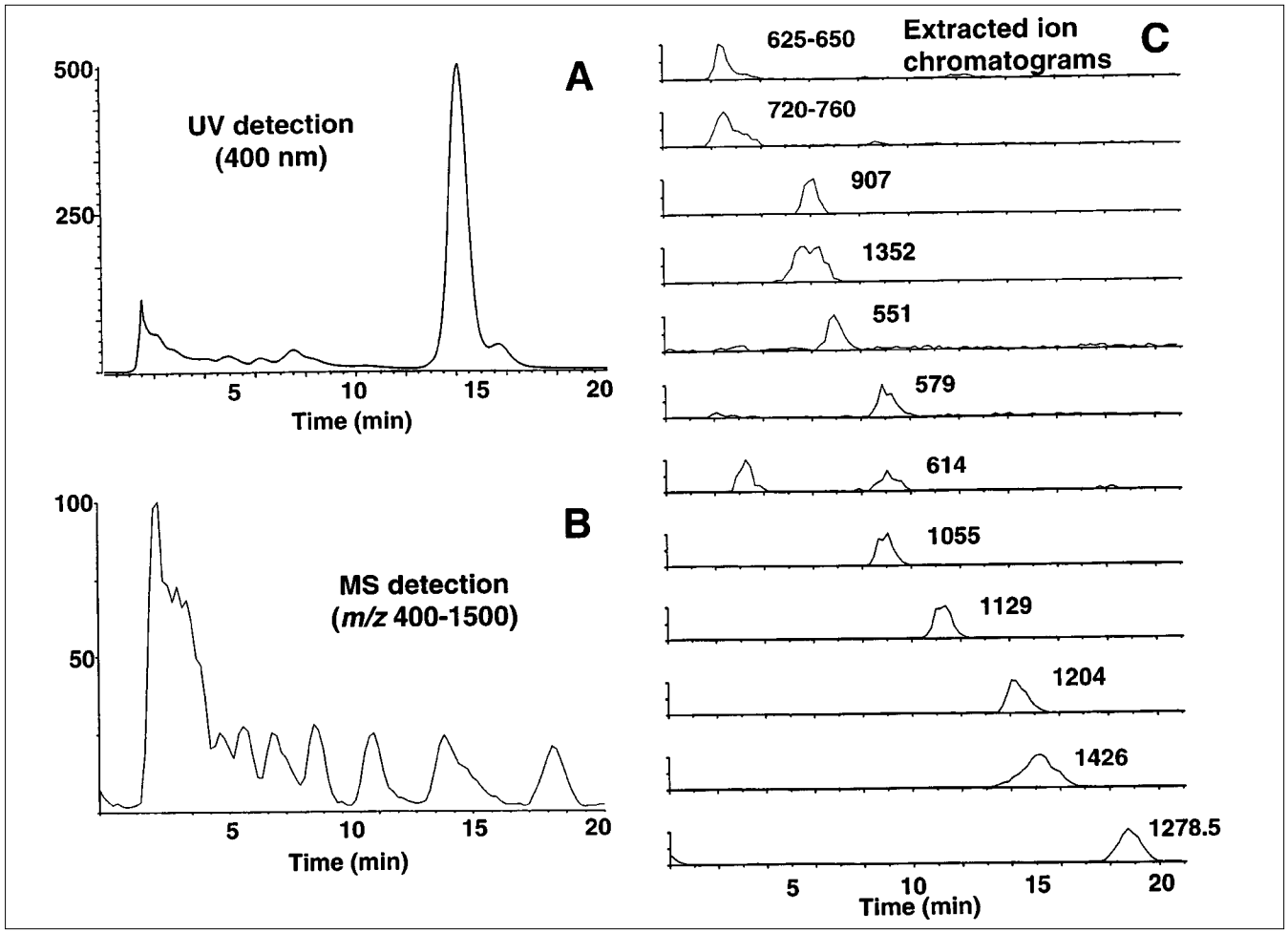


FIG. 4. High-performance liquid chromatography ultraviolet–(UV) mass spectrometry–(MS) analysis of β-carotene/phenol/horseradish peroxidase/H_2O_2 reaction mixture quenched by cooling to 0°C. (A): UV-detected (400 nm) chromatogram of products. Note that β-carotene had a retention time of 7.5 min in this system. (B): Total ion chromatogram (m/z 400-1500) of products. The large, early-eluting signals were also evident in control reaction mixtures (time = *ca.* 0). (C): Extracted ion chromatograms of some of the ion signals detected from the reaction products. Note the lack of signals in the ranges *m/z* 625–650 and *m/z* 720–760 where phenol adducts of β-carotene are expected to appear.

of ascorbate on β-carotene consumption by HRP/H_2O_2 in the presence of phenol or PMC. Since phenol oxidation products were apparently not involved in the degradation of β-carotene, we further tested the hypothesis that the phenoxyl radical (or similar intermediates) was responsible for its oxidation. The phenoxyl radical is very short-lived and cannot be detected directly by conventional ESR (33). We therefore documented the participation of the phenoxyl radical in the oxidation of β-carotene by using ascorbate, which is highly reactive toward phenoxyl radicals (34). In the absence of ascorbate, incubation media containing β-carotene "micelles" and HRP/H_2O_2 did not produce any ESR-detectable signals (Fig. 5). Incubation of ascorbate with HRP/H_2O_2 generated a weak semidehydroascorbyl radical ESR signal, the intensity of which dramatically increased upon addition of phenol. In the absence of phenol, the weak semidehydroascorbyl radical ESR signal could be observed for 50–60 min without any change in its magnitude. Addition of phenol, on the other hand, caused an almost immediate increase in the intensity of the semidehydroascorbyl signal that, under the conditions used, completely disappeared from the spectra after 30 min of incubation. Importantly, we found that oxidation of β-carotene by HRP/H_2O_2/phenol was prevented by ascorbate during the period when the semidehydroascorbyl radical was discernible in ESR spectra (Fig. 6).

Peroxidase/HRP/H_2O_2-induced oxidation of PMC in the presence of β-carotene was accompanied by the formation of the characteristic five-line ESR signal of PMC phenoxyl radical (35). Addition of ascorbate resulted in the disappearance of the PMC phenoxyl radical signal and appearance of the characteristic doublet semidehydroascorbyl radical signal (Fig. 5).

Effects of phenol and PMC on oxidation of β-carotene induced by azo-initiators (AMVN and AAPH) in DOPC-liposomes. β-Carotene can be oxidized by peroxyl radicals, and this oxidation can be prevented by phenolic antioxidants (22). If phenoxyl radicals formed by reaction of a phenolic compound with a peroxyl radical are reactive enough to directly oxidize β-carotene, however, no protection should be granted by the phenolic compound. We used hydrophilic and hydrophobic azo-initiators of peroxyl radicals, AAPH and AMVN, respectively, to study the effects of phenol and PMC on the oxidation of β-carotene in DOPC-liposomes (Fig. 7). We found that both AAPH and AMVN caused oxidation of β-carotene. PMC protected β-carotene against oxidation induced by either azo-initiator. In contrast, phenol did not inhibit AAPH- or AMVN-induced oxidation of β-carotene. Moreover, a weak but enhanced oxidation of β-carotene occurred in the presence of phenol when hydrophobic AMVN was used to initiate peroxyl radical formation.

Effect of phenol on the consumption of different carotenoids in model systems by HRP/H_2O_2. To test whether the results we had observed were unique to β-carotene or could also be extended to the oxidation of other, similar compounds, we studied the effects of phenol on the consumption of several representative carotenoids by HRP/H_2O_2 (Table 2). We found that not only β-carotene, but also canthaxanthin, lutein and lycopene were readily oxidized by this system. In the presence of phenol, the oxidation rate constants for canthaxanthin and lutein were even higher than that for β-carotene. Oxidation rates were 2- to 4-fold lower in the absence of phenol. While lycopene had a lower oxidation rate both in the presence and in the absence of phenol, the conditions under which it was studied were necessarily different from those used for the other carotenoids. Since we could not

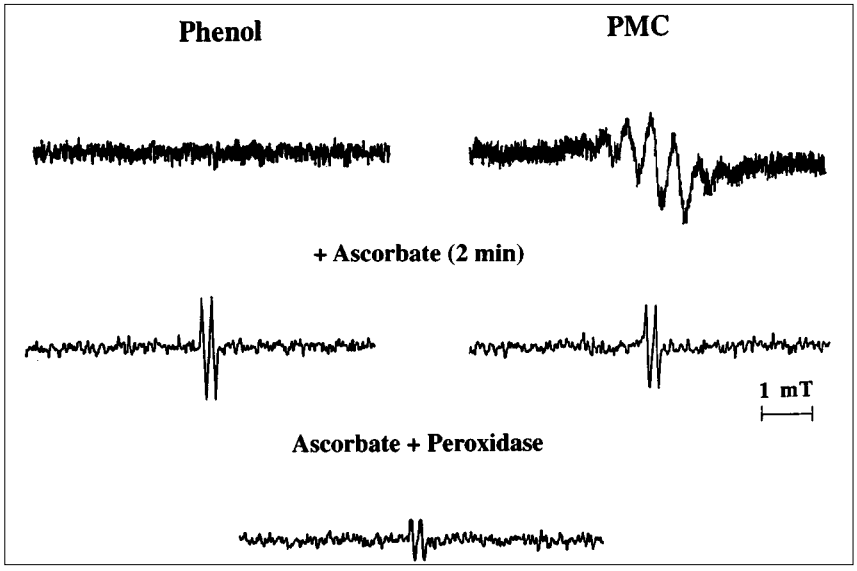


FIG. 5. Electron spin resonance spectra of PMC phenoxyl radical and semidehydroascorbyl radical induced by horseradish peroxidase/H_2O_2 in "micelles" of β-carotene. Reaction conditions: incubation was performed in phosphate buffer (50 mM) containing NaCl (100 mM), pH 7.4, with β-carotene (20 μM), HRP (0.1 U/mL)/H_2O_2 (400 μM) and phenol (100 μM) or PMC (100 μM). See Figure 1 for other abbreviation.

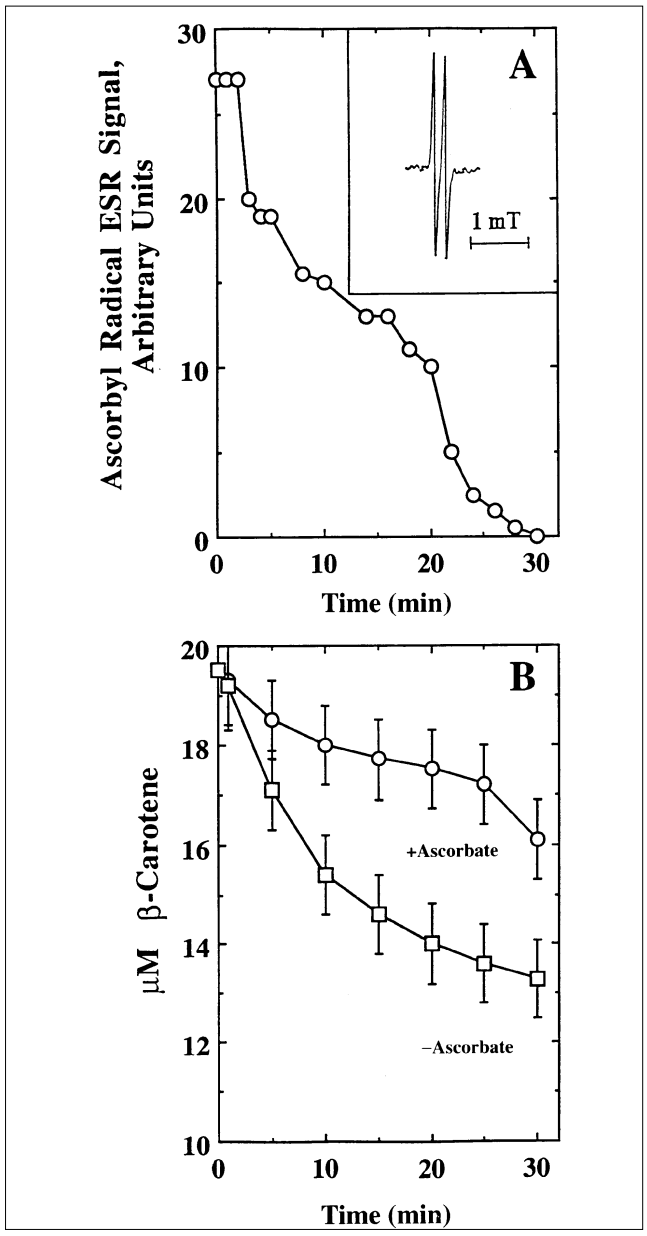


FIG. 6. Time course of semidehydroascorbyl radical (A) and β-carotene oxidation (B) by horseradish peroxidase-catalyzed oxidation of phenol. The electron spin resonance (ESR) spectra spectrum of semidehydroascorbyl radical is shown (A, insert). Reaction conditions: β-carotene added to phosphate buffer (50 mM) containing NaCl (100 mM), pH 7.4, was incubated at 25°C with HRP (0.1 U/mL)/H_2O_2 (400 μM) and phenol (100 μM) either in the absence (□) or presence (○) of ascorbate (30 μM).

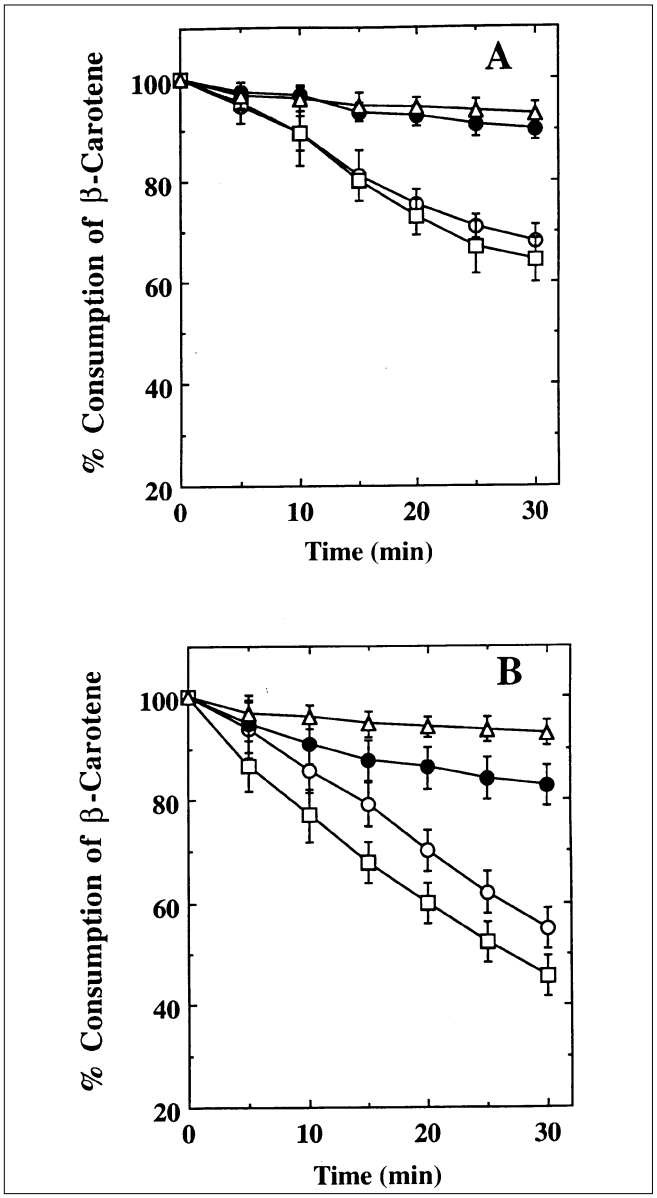


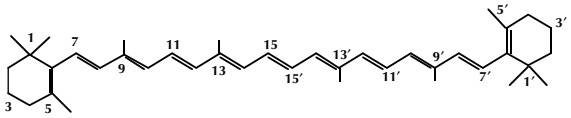
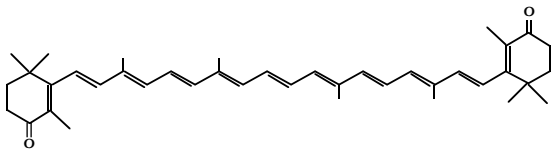
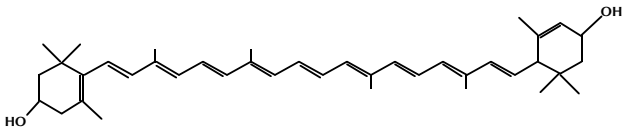
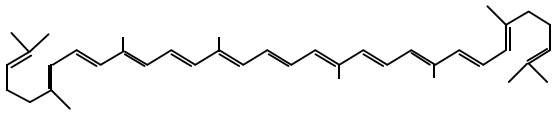
FIG. 7. Effect of phenol and PMC on β-carotene oxidation in 1,2,-diolerylphosphatidylcholine (DOPC) liposomes in the presence of 2,2'-azo-*bis* (2-amidinopropane)dihydrochloride (AAPH) (A) or 2,2'-azo-*bis*(2,4-dimethylvaleronitrile) (AMVN) (B). Reaction conditions: liposomes of DOPC (510 μM) and β-carotene (20 μM) were incubated in phosphate buffer (50 mM) containing NaCl (100 mM), pH 7.4, at 37°C with: (A) AAPH (50 mM) (○), phenol (100 μM) and AAPH (50 mM) (□), or PMC (100 μM) and AAPH (50 mM) (●); or with (B) AMVN (1 mM) (○), phenol (100 μM) and AMVN (1 mM) (□), or PMC (100 μM) and AMVN (1 mM) (●), or without additions (△). See Figure 1 for other abbreviation.

find conditions useful for the preparation of fine homogenous "micelles" of lycopene in phosphate buffer, we integrated it into DOPC liposomes and then performed incubations in the presence of HRP (0.5 U/mL) and H_2O_2 (400 μM).

DISCUSSION

The unsaturated carbon-carbon bonds of membrane lipids are the primary targets for free radicals reactions *in vivo*. Their consequent peroxidation results in membrane damage (36). In addition to reductive enzymes, the antioxidant defense system includes water- and lipid-soluble free radical scavengers (vitamin C, thiols, vitamin E, coenzyme Q, and carotenoids) whose activities are enhanced by synergistic interactions through their recycling mechanisms (34,37,38). In particular, the importance of redox-cycling cascades such as thiols → ascorbate → tocopherol and NAD(P)H → electron transport

TABLE 2
Rate Constant of Carotenoid Oxidations by Peroxidase/H_2O_2

Carotenoids	Rate constants, K (10^{-4} s^{-1})		Structures of carotenoids
	− Phenol	+ Phenol	
β-Carotene[a]	2.1 ± 0.1	4.4 ± 0.3	
Canthaxanthin[a]	1.6 ± 0.1	6.2 ± o.4	
Lutein[a]	2.2 ± 0.1	5.4 ± 0.3	
Lycopene[b]	0.9 ± 0.1	2.1 ± 0.1	

[a]Incubation mixtures contained micelles of carotenoids prepared by addition of tetrahydrofuran solutions of the respective compound to 50 mM phosphate buffer, pH 7.4, containing peroxidase (0.1 U/mL), H_2O_2 (400 μM), and with or without phenol (100 μM).
[b]Since lycopene could not be homogeneously dispersed in the phosphate buffer, it was integrated in 1,2-dioleoylphosphatidylcholine liposomes and then incubated in the presence of peroxidase (0.5 U/mL) and H_2O_2 (400 μM) with or with phenol (100 μM).

enzymes → coenzyme Q → vitamin E has been established (39). In several epidemiological studies, dietary consumption of carotenoids has been correlated with a decreased risk of cancer at several tissue sites (40); yet, a statistically significant increased lung cancer incidence (18%) has been reported in male smokers (41). While carotenoids are known to be precursors of vitamin A (42), their important protective properties, such as enhancing the immune response, inhibiting mutagenesis, reducing tumor development and causing tumor regression *in vivo*, lowering cell transformation *in vitro*, and preventing sister chromatid exchange, have been associated with their antioxidant function (20,43), independent of their metabolism to vitamin A (44). Antioxidant mechanisms of carotenoids include effective quenching of singlet oxygen (40) as well as chainbreaking scavenging of carbon-centered and oxygen radicals, which is efficient at low oxygen pressures (21–24).

The antioxidant activity of carotenoids is dependent on their concerted action with other chainbreaking lipid-soluble antioxidants (44). In particular, phenolic antioxidants, vitamin E and coenzyme Q, act to protect carotenoids against oxidative destruction (45). For example, in LDL exposed to a steady flux of aqueous peroxyl radicals, ubiquinol-10 is the first antioxidant to be consumed, followed by α-tocotrienol and α-tocopherol, all of which disappear before the carotenoids are consumed (46). If the reactivity of phenoxyl radicals formed from phenolic antioxidants is high, phenoxyl radical-induced oxidation of carotenoids, rather than their protection by phenolic compounds, is likely to occur. Recently, Bowry *et al.* (47) suggested that phenoxyl radicals of vitamin E may be responsible for oxidation of lipids in LDL, at least under some conditions.

Our previous work demonstrated that phenoxyl radicals generated from phenol and the phenolic antitumor drug etoposide (VP-16) induce one-electron oxidation of protein sulfhydryls and GSH in cells and model systems, subsequently generating thiyl radicals, disulfide anion radicals, and superoxide (in the presence of molecular oxygen), thus triggering the chain of events ultimately resulting in oxidative stress (8,13). Oxidation of thiols was not observed when phenoxyl radicals were generated from vitamin E or its homologues (13). To further understand the reactions of phenoxyl radicals with physiologically important molecules, in the present work we used two representative phenolic compounds, phenol and PMC, to study interactions of their respective phenoxyl radicals toward carotenoids.

Myeloperoxidase-catalyzed oxidation of β-carotene in HL-60 cells. Our results demonstrated that β-carotene associated with HL-60 cells was oxidized by phenol/H_2O_2, but not by either agent alone. HL-60 cells contain very high myeloperoxidase activity (48). Since azide inhibited oxidation of phenol (i.e., inhibited peroxidase activity) and simultaneously inhibited oxidation of β-carotene, we suggest that oxidation of β-carotene was catalyzed by endogenous myeloperoxidase.

While inhibition by azide is not strictly specific for myeloperoxidase and may involve other hemoproteins, myeloperoxidase is the most abundant hemoprotein in HL-60 cells (48).

In contrast to phenol, PMC/H_2O_2 was ineffective in oxidizing β-carotene. One possible explanation for this difference may be that myeloperoxidase-catalyzed oxidation of these two phenolic compounds proceeds at dramatically different rates. Our direct measurements showed that phenol was oxidized at a rate twice that of PMC. Thus, the qualitative difference—fast oxidation of β-carotene in the presence of phenol vs. lack of its oxidation in the presence of PMC—can hardly be explained by differences in the kinetics of the myeloperoxidase-catalyzed oxidation of phenol and PMC.

Previous studies show that leukocyte myeloperoxidase causes a rapid destruction of β-carotene in the presence of H_2O_2/halides (49). The myeloperoxidase/H_2O_2/halide system produces potent oxidants, such as hypochlorous acid and singlet oxygen (50), that are known to readily oxidize different lipids (including carotenoids) (51) and proteins. (52). The mechanism of myeloperoxidase/H_2O_2/halide-induced oxidation of carotenoids is likely to differ from that of myeloperoxidase-catalyzed oxidation in the presence of H_2O_2 and phenolic compounds. The peroxidase-catalyzed release of phenoxyl radicals has been shown to induce oxidation of critical biomolecules and thus cause cytotoxic effects (28,53). The myeloperoxidase-catalyzed activation of phenol and other phenolic metabolites of benzene (e.g., hydroquinone, benezenetriol) in bone marrow is often invoked as the mechanism responsible for benzene-induced aplastic anemia and leukemia (5,54,55). The reactivity of phenoxyl radicals toward lipid-soluble antioxidants and lipids, causing the formation of mutagenic lipid peroxidation products, may contribute to the carcinogenicity of benzene. To the best of our knowledge, we have demonstrated for the first time that intracellular myeloperoxidase-catalyzed oxidation of phenol in the presence of H_2O_2 results in oxidation of a carotenoid, i.e., depletion of an important lipid-soluble antioxidant. While it is conceivable that phenoxyl radicals generated by myeloperoxidase in HL-60 cells were responsible for the oxidation of incorporated β-carotene, an explanation should exist to explain the different effects of phenol and PMC. To this end we performed experiments in simple model systems where HRP/H_2O_2 and phenol or PMC were used to oxidize β-carotene in "micelles."

Phenoxyl radicals directly oxidize β-carotene in the HRP/H_2O_2/phenol system. Our model experiments clearly demonstrated that oxidation of β-carotene by HRP/H_2O_2/phenol was not due to phenol oxygenation products (e.g., quinones), but rather involved reactions with free radical intermediates. Specifically, phenoxyl radicals of phenol (but not of PMC) directly oxidized β-carotene in a reaction catalyzed by HRP in the presence of H_2O_2. The reaction was inhibited by ascorbate, and this inhibition was accompanied by generation of semidehydroascorbyl radical, a one-electron product of ascorbate oxidation. Previous studies have shown that ascorbate is an effective one-electron reductant for different phenoxyl radicals (34,37,38). The phenoxyl radical of PMC, which is directly observable by ESR, was also reduced by ascorbate to produce the identical characteristic ESR signal of semidehydroascorbyl radical. These results suggest that different relative reactivities of phenoxyl radicals toward β-carotene may be responsible for the different behaviors of phenol and PMC.

Since the relative reactivities of phenoxyl radicals may govern the protective (antioxidant) vs. toxic effects of phenolic radical scavengers, we subsequently compared the effects of phenol and PMC on oxidation of β-carotene induced by water- and lipid-soluble azo-initiators of peroxyl radicals (56). We found that PMC protected β-carotene against oxidation induced by either AAPH or AMVN. In both cases, phenol afforded no protection; rather, it enhanced the oxidation of β-carotene. These results fully concur with our previous findings on the protection of proteins against oxidation by vitamin E homologues and, vice versa, the prooxidant effects of phenol (*via* its phenoxyl radical) in both cells and in model systems.

Recently, Liebler and McClure (31) provided direct proof for the antioxidant mechanism of β-carotene by demonstrating formation of carotenoid–radical adducts when oxidation was induced by thermolysis of AMVN. We found no evidence of direct reaction of phenoxyl radicals with β-carotene or β-carotenyl radicals. This suggests that β-carotene is not likely to terminate phenoxyl radical-induced oxidation reactions, at least not those catalyzed by peroxidase. Given that the peroxidase-catalyzed reaction is considered an important and physiologically-relevant oxidation mechanism in plasma (15), our results suggest that β-carotene does not seem a likely candidate for protection of LDL against oxidative stress.

Finally, the prooxidant effects of the phenol phenoxyl radical are not limited to β-carotene, but may embrace other carotenoids (e.g., lycopene, lutein, canthaxanthin). While the results presented in the study were obtained with phenol, intracellular phenoxyl radicals generated from other phenolic compounds may be involved in the oxidative degradation of carotenoids. In particular, tyrosine phenoxyl radical (TyrO·) has been detected recently in a number of proteins (57). Calculated spin densities for the respective phenoxyl radicals of phenol and tyrosine differ from each other by less than 0.03, implying that the unpaired electron of TyrO(·) resides entirely on the aryloxyl portion of the molecule. Since carotenoids are essential constituents of the antioxidant defenses used by cells and biological fluids (42), their depletion through the reaction with phenoxyl radicals formed from endogenous, nutritional and environmental phenolics (e.g., estrogens, flavonoids, polyphenols, chlorinated phenols) or phenolic drugs (e.g., etoposide, acetaminophen) may be an important factor in the development of oxidative stress. Not surprisingly, peroxidase-catalyzed oxidation of LDL is now considered an indispensable model in studies of oxidative mechanisms of atherosclerosis (17). The question remains whether other polyunsaturated lipids (e.g., methylene-interrupted fatty acid residues of membrane phospholipids) may also be involved in oxidations by phenoxyl radicals generated from different phenolic

compounds.

NOTE ADDED IN PROOF

In a recent paper, Mortensen and Skibsted [*Free Radical Res. 25*:515–523 (1996)] present evidence for the formation of adducts between phenoxyl radicals generated by laser flash photolysis and β-carotene. These conditions (when both phenoxyl radicals and β-carotenyl radicals might be simultaneously present and thus directly interact with each other) are distinctly different from those used in our experiments (peroxidase-catalyzed phenoxyl radicals could only attack β-carotene).

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Stearic Acid Modifies Very Low Density Lipoprotein Lipid Composition and Particle Size Differently from Shorter-Chain Saturated Fatty Acids in Cultured Rat Hepatocytes

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ABSTRACT: Stearic acid as compared to myristate, palmitate, or oleate is poorly incorporated into triacylglycerol, a major lipid component of very low density lipoprotein (VLDL). The present study investigated the effects of these fatty acids on VLDL metabolism in cultured rat hepatocytes. All fatty acids stimulated [2-^{3}H] glycerol incorporation into VLDL lipids and secretion of [^{3}H]-labeled VLDL by hepatocytes. However, the rate of [^{3}H]-labeled VLDL secretion in the presence of nonlabeled stearate (12.8 ± 0.7 pmol/mg protein/4 h) was 46, 59, and 22% of that observed for those treated with myristate, palmitate, and oleate, respectively. [1-^{14}C]Stearate as a substrate was also less effective than other labeled fatty acids to be incorporated into VLDL lipids. Of total VLDL lipids synthesized from [1-^{14}C] stearate, triacylglycerol accounted for 78% as compared to 88–97% of that derived from palmitate, myristate, and oleate. The amounts of apoB100 and apoB48 were the same in hepatocytes treated with or without exogenous fatty acids. Similarly, the rate of apoB synthesis from [^{35}S] methionine was not affected by exogenous fatty acids. The treatment of cells with various saturated fatty acids increased the particle size of VLDL to different extents. The largest particles of VLDL, with a mean diameter of 79.3 ± 11.9 nm, were seen in the cells treated with stearate, followed by those treated with palmitate and myristate (45.5 ± 9.8 and 38.6 ± 6.8 nm, diameter, respectively). Clearly, hepatocytes treated with stearate secrete less VLDL and produce larger VLDL particles than those treated with shorter-chain saturated fatty acids.

Lipids 32, 143–149 (1997).

The neutral effect of stearate on plasma cholesterol concentration is well defined (1–5). Despite the effect, current diet recommendations do not differentiate stearate from other saturated fatty acids in their influence on the plasma cholesterol level (6,7). This may be attributed in part to a lack of complete understanding of the mechanism(s) underlying the neutral effect of stearate, although poor intestinal absorption of stearate, (8) desaturation of stearate to oleate (9), and absence of inhibitory effect of stearate on low density lipoprotein (LDL)-receptor activity (10) have been proposed as possible explanations. The roles of dietary saturated and polyunsaturated fats in regulating plasma cholesterol level and LDL metabolism have been extensively investigated in animals and humans (11,12). However, the importance of individual fatty acid in cholesterol metabolism, especially very low-density lipoprotein (VLDL) synthesis and secretion, has not been established. Since plasma VLDL is a precursor of LDL, any alteration in VLDL metabolism is bound to change LDL concentration (13). Physical and biochemical characteristics of lipoproteins are known to be altered by saturated and unsaturated fatty acids in the diet. For example, chylomicrons are smaller in rats fed a diet high in saturated fatty acids than those fed a diet high in unsaturated fatty acids (14). Larger sized chylomicrons and VLDL are cleared faster than the smaller ones from plasma (15,16). Fish oil rich in eicosapentaenoic and docosahexaenoic acids is effective in reducing plasma level of triacylglycerol due primarily to a decrease of hepatic VLDL-triacylglycerol secretion in humans (17). Fish oil has also been shown to increase the degradation of apoB in the rat hepatocytes (18).

We have previously shown that stearate treatment markedly inhibited triacylglycerol synthesis in cultured rat hepatocytes (19). Since triacylglycerol is a major component of VLDL (20,21), it is conceivable that the impaired triacylglycerol synthesis could decrease VLDL synthesis and secretion. The present study was, therefore, designed to evaluate the effects of various saturated fatty acids on VLDL secretion as well as chemical and physical properties of VLDL particles. The results showed that stearate compared to other saturated fatty acids not only reduced VLDL secretion but also increased the particle size of VLDL.

MATERIALS AND METHODS

Animals and diets. Sprague-Dawley male rats (100–150 g body weight), obtained from Harlan Sprague-Dawley (Indi-

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Abbreviations: apoB, apolipoprotein B; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

anapolis, IN), were fed *ad libitum* rat chow (Ralston Purina Co., St. Louis, MO) and had free access to tap water. All animals were housed individually in steel-mesh cages in a room with controlled temperature and lighting (22°C and 12 h light–dark cycle). The animal protocol was approved by the Pennsylvania State University Institutional Animal Care and Use Committee (University Park, PA).

Hepatocyte isolation and culture. Hepatocytes were isolated from rats (225–250 g body weight) by the method of Berry and Friend (22) as modified by Seglen (23). As detailed elsewhere (24), the procedure involved collagenase perfusion *via* hepatic portal vein, tissue mincing, filtration and centrifugation, and further purification by cell adherence. The isolated cells were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 5.6 mmol/L glucose, 10% fetal bovine serum (FBS), and antibiotics (penicillin, 100 units/mL and streptomycin, 100 μg/mL). Cell viability was determined by trypan blue exclusion for each isolation. Cell preparations with 95% or higher viability were used throughout the experiments. A 2-mL aliquot of cell suspension ($0.7–0.8 \times 10^6$ cells/mL) was transferred to culture plates, each containing six wells (3.5 cm diameter), and incubated at 37°C under an atmosphere of 95% air and 5% CO_2. Six hours after incubation, cells adhering to the flask were refed with DMEM and incubated under the same conditions for 24 h.

Synthesis and isolation of VLDL. The cultured cells obtained after 24 h incubation were washed three times with 2 mL of FBS-free DMEM followed by incubation in the same medium containing [2-^{3}H]glycerol (specific activity of 7.4 MBq/mmol) at 25 μmol/L in the presence or absence of one of the unlabeled fatty acids at 0.5 mM. Fatty acids were provided as an albumin complex in a molar ratio of 5.6:1 (fatty acid/albumin). Stock solutions (100 mmol/L) were prepared from the sodium salt of each fatty acid. An aliquot of the stock solution was added to DMEM containing 0.58% bovine serum albumin and sonicated prior to incubation with hepatocytes.

At the end of 4 h incubation, the medium (2 mL) was transferred to centrifuge tubes for isolation of VLDL (25). The density of the medium was adjusted to 1.21 g/mL by adding 1.3 g potassium bromide and 2 mL saline. Salt solution with a density of 1.0063 g/mL (1.15% NaCl (wt/vol)) was carefully layered over and centrifuged at $100{,}000 \times g$ for 22 h. After the centrifugation, VLDL on the top of the solution was collected, and VLDL lipid was extracted according to the method of Folch *et al.* (26). The radioactivity of VLDL lipids was measured by liquid scintillation spectrometry (LS8100; Beckman Instruments Inc., Palo Alto, CA). The rate of glycerol incorporation into VLDL lipids was expressed as pmol glycerol incorporated/mg protein/4 h. The rate of VLDL secretion was measured by [2-^{3}H]glycerol recovered in the medium VLDL (27).

Apolipoprotein B synthesis. Cultured hepatocytes were incubated with 2 mL of FBS-free DMEM containing 0.2 mM [^{35}S]methionine (specific activity of 0.82 MBq/mmol). After 4 h incubation, apolipoprotein B(apoB) was immunoprecipitated by the method of Wang *et al.* (18). ApoB was separated by electrophoresis in a 3.5% polyacrylamide–18% glycerol gel (28). After fixing and staining the gel, the density of each band was measured by using a laser densitometer to determine the mass of apoB (29). The amount of apoB determined for hepatocytes treated with exogenous fatty acids was expressed as percentage of density detected in the gel of control hepatocytes. After the densitometeric measurements, each band was excised from the gel and dissolved in 35% H_2O_2 overnight. The radioactivity of each band was measured by liquid scintillation counting. The rate of [^{35}S]methionine incorporation into apoB was expressed as cpm/mg protein/4 h.

Synthesis of VLDL lipids. Cultured cells obtained after 24 h incubation were washed three times with 2 mL of FBS-free DMEM and were incubated in FBS-free medium containing 0.5 mmol/L [9,10-^{3}H]myristate, [1-^{14}C]palmitate, [1-^{14}C]-stearate, or [1-^{14}C]oleate, and nonradioactive fatty acid with a specific activity of 37 MBq/mmol. After 4 h incubation, VLDL was isolated as above, and VLDL lipids were extracted. The extracted lipids were separated into phospholipid, triacylglycerol, diacylglycerol, monoacylglycerol, and cholesterol esters by thin-layer chromatography (30). Bands containing the individual lipid classes were scraped from the plate and reextracted with chloroform/methanol (2:1; vol/vol). The radioactivity derived from lipid classes was measured as described above. The rate of lipid synthesis was expressed as nmol substrate incorporated/mg protein/4 h.

VLDL particle size measurements. Cultured hepatocytes were incubated with 2 mL of FBS-free DMEM containing individual fatty acids (0.5 mmol/L) and glycerol (25 μmol/L) and processed according to the procedure described above for synthesis of VLDL. After the VLDL isolation by gradient centrifugation, VLDL was negatively stained with 2% phosphotungstate (pH 7.4) and viewed in a Jeol 1200EXII transmission electron microscope (Jeol USA, Peabody, MA) at 60 KeV. The diameter of VLDL particle was measured by using Image Archival Imix, version seven (PGT, Princeton, NJ). Briefly, the transmission electron microscope images of VLDL were electronically transferred to the Image Archival Imix program, and the size of VLDL was determined by measuring the diameter of each VLDL particle with a laser digitizer.

Materials. Chemical reagents were purchased from Sigma Co. (St. Louis, MO) unless otherwise indicated. Collagenase D was purchased from Boehringer Mannheim Co. (Indianapolis, IN). [2-^{3}H]Glycerol, [9,10-^{3}H]myristate, [1-^{14}C]palmitate and [1-^{14}C]stearate, [1-^{14}C]oleate, [1-^{14}C]linoleate, and [^{35}S]methionine were obtained from Amersham Co. (Arlington Heights, IL). DMEM, FBS and antibiotics (penicillin and streptomycin) were products of Life Technologies, Inc. (Grand Island, NY). Silica gel H plates (20×20 cm, 250 μm layer thickness) were supplied by Alltech Associates, Inc. (Deerfield, IL).

Statistical analysis. Data are expressed as mean $\pm$ SD. Results from the multiple fatty acid treatments were compared by analysis of variance. Duncan's Multiple Range Test was applied to identify the significant differences at $P < 0.05$.

RESULTS

The effects of fatty acids on VLDL secretion were determined by measuring the rate of [2-^{3}H] glycerol incorporation into lipids of VLDL detected in the culture medium. The rates of [2-^{3}H]glycerol incorporation were 12.8 ± 0.7, 18.6 ± 2.7, 29.7 ± 3.8, and 63.7 ± 2.6 pmol/mg protein/4 h in the hepatocytes treated with stearate, palmitate, myristate, and oleate, respectively, compared to 6.6 ± 0.3 pmol/mg protein/4 h seen in the control untreated hepatocytes. Clearly, all fatty acids markedly stimulated [2-^{3}H]glycerol incorporation into VLDL lipids (Fig. 1). However, the percentage of stimulation was the lowest by stearate (195%) and the highest by oleate (970%). Interestingly, the degree of stimulation by saturated fatty acids was inversely related to the carbon chain length. The secretion of VLDL was also determined by measuring the incorporation of [^{3}H]- or [^{14}C]-labeled fatty acids into VLDL lipids recovered in the culture medium. The rate of [1-^{14}C]stearate incorporation into VLDL total lipids was 16, 16, and 6% of that for palmitate, myristate, and oleate, respectively (Table 1). The rate of [1-^{14}C] oleate incorporation was the highest among all fatty acids. Regardless of the labeled fatty acid substrates, triacylglycerol was the predominant product. Thus, the differential effects of fatty acids on total lipid synthesis resulted primarily from differences in their incorporation into triacylglycerol. The rate of phospholipid synthesis was the highest from oleate and palmitate, and the lowest from stearate. The rate of palmitate incorporation into diacylglycerol was higher than that of other fatty acids. However, there were no differences in the incorporation among stearate, myristate, and oleate (Table 1). The rate of stearate incorporation into cholesteryl ester was markedly lower than that of oleate, palmitate, and myristate. Consequent to the disproportional incorporation of fatty acids into various lipids, of the radioactivity recovered in total lipids, triacylglycerol accounted for 97, 96, 88, and 78% from labeled oleate, myristate, palmitate, and stearate, respectively. The reduced percentage of triacylglycerol from stearate was accompanied by slight increases in phospholipid and diacyl-

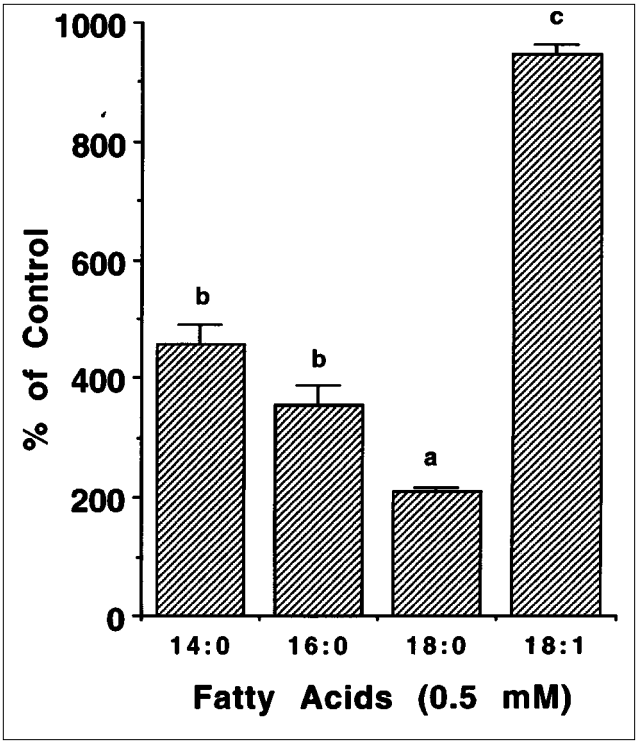

FIG. 1. Incorporation of [2-^{3}H]glycerol into very low density lipoprotein (VLDL) as affected by exogenous fatty acids in cultured rat hepatocytes. Values are mean ± SD for four samples. [2-^{3}H]Glycerol was incubated with 0.5 mM fatty acid for 4 h. Percentage of control is defined as the amount of [^{3}H] lipid radioactivity recovered in VLDL secreted by individual fatty acids treated hepatocytes divided by that secreted by hepatocytes without any fatty acid treatment, multiplied by 100.

TABLE 1
Incorporation of [^{3}H] or [^{14}C]-Labeled Fatty Acids into Lipids of VLDL Secreted by Cultured Rat Hepatocytes[a]

	Labeled substrate			
	Myristate	Palmitate	Stearate	Oleate
pmol substrate incorporation/mg protein/4 h				
Phospholipids	22 ± 7^a	39 ± 9^b	13 ± 6^a	38 ± 3^b
Diacylglycerols	12 ± 2^a	42 ± 3^b	20 ± 7^{a,c}	30 ± 9^c
Triacylglycerols	1274 ± 170^a	1101 ± 279^a	138 ± 53^b	2724 ± 787^c
Cholesteryl esters	20 ± 1^a	66 ± 33^b	5 ± 2^c	21 ± 3^a
Total[b]	1368 ± 201^a	1305 ± 321^a	210 ± 61^b	3270 ± 607^c
% Distribution				
Phospholipids	1.7 ± 0.9^a	3.1 ± 0.8^b	7.3 ± 0.2^c	1.3 ± 0.2^a
Diacylglycerols	0.9 ± 0.1^a	3.4 ± 0.8^b	11.4 ± 0.2^c	1.0 ± 0.5^a
Triacylglycerols	95.8 ± 0.9^a	88.2 ± 3.8^b	78.4 ± 1.0^c	97.0 ± 0.9^a
Cholesteryl esters	0.5 ± 0.1^a	5.2 ± 0.8^b	2.8 ± 0.5^c	0.7 ± 0.1^a

[a]Values are means ± SD for four samples. Hepatocytes were incubated with 0.5 mM [^{3}H]- and [^{14}C]-labeled fatty acid for 4 h. Very low density lipoprotein (VLDL) in the medium was isolated for analysis of lipids by thin-layer chromatography and liquid scintillation spectrometry. Values with different superscript letters in the same row are significantly different at $P < 0.05$.

[b]Total represents the sum of the four lipid classes listed and other minor lipids such as free cholesterol, free fatty acids, and monoacylglycerol.

glycerol. However, the percentage of cholesteryl ester from palmitate was higher than that from stearate (Table 1).

The particle size of VLDL was measured in subsequent experiments. The VLDL particles secreted by hepatocytes of control group had the smallest mean diameter (35.6 ± 4.8 nm diameter) as compared to other groups (Fig. 2). The largest particles with a mean diameter of 79.3 ± 11.9 nm were obtained from the cells treated with stearate. The mean particle diameters for the cells treated with palmitate and myristate were 45.5 ± 9.8 and 38.6 ± 6.8 nm, respectively. The mean diameter of VLDL (71.6 ± 15.6 nm) of oleated-treated cells was comparable for that of stearate-treated counterparts. The frequency distribution of VLDL from oleate could not be determined because of insufficient number of particles. The distribution of VLDL particle size from control and myristate-treated cells was similar with the range falling between 25 and 55 nm in particle diameter. The peak frequencies fall at 35 nm from both groups, but the peak frequency represents 41% for the control and 28% for the myristate-treated group. The VLDL particles derived from palmitate-treated cells were distributed over a wider range (20 to 75 nm in diameter). There was no sharp peak in frequency in this group; however, 61% of particles fall between 40 and 50 nm range. The distribution of the particles derived from stearate-treated cells falls beyond the range of those obtained from control, myristate, and palmitate-treated cells, although there was some overlap with the palmitate-treated group. The particle size of stearate-treated cells ranged from 55 to 105 nm in diameter with greater than 58% of the population falling between 75 and 90 nm.

The results of apoB experiments are presented in Table 2. The relative content of apoB was determined on the basis of the apoB density on the gel prepared from the cells treated without exogenous fatty acids. The treatment of the cells with fatty acids did not alter apoB100 and apoB48 contents measured in the cells and the culture medium (Table 2). Approximately 40–50% of apoB100 and apoB48 was released into the medium presumably in the form of VLDL. The rate of apoB synthesis was measured using [^{35}S]methionine as a substrate. The recoveries of radioactivity in apoB48 in the cells and the medium were not altered by the exogenous fatty acids. Similarly, fatty acids did not affect the incorporation of [^{35}S]-labeled methionine incorporation into the apoB100 detected in the medium. However, stearate, palmitate, and oleate decreased the radioactivity recovery in the cellular apoB100 (Table 2). When the amounts of radioactivity recovered in both the cells and the medium were added together, the rate of synthesis of apoB100 and apoB48 did not differ among the groups treated with or without the fatty acids. Approximately 82–87% of the newly synthesized apoB100 was secreted from the cells into the medium, whereas 62–74% of the apoB48 was secreted, and the secretions of both were not affected by the fatty acids present in the incubation medium.

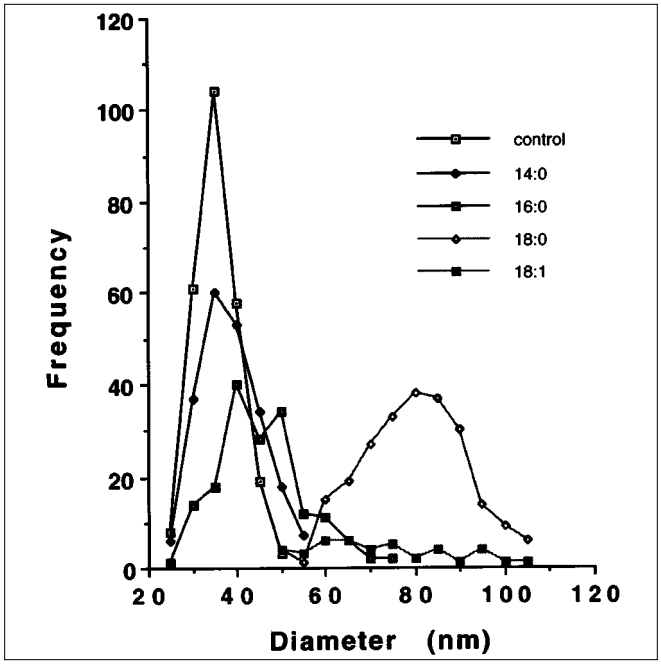

FIG. 2. Particle size distribution of VLDL secreted by hepatocytes treated with saturated fatty acids. After 4 h incubation of the cells with 0.5 mM fatty acid, VLDL in the medium was isolated. Percentage distribution is defined as the percentage of VLDL particles of which the diameters fall within the 5-nm interval. A total of 253, 215, 168, and 231 VLDL particles obtained from the cells treated with no fatty acid, myristate, palmitate or stearate, respectively, were included in the calculation. See Figure 1 for abbreviation.

DISCUSSION

The production of VLDL, a triacylglycerol-rich lipoprotein, takes place in the liver and is dependent on the availability of triacylglycerol, phospholipid, free and esterified cholesterol, and apolipoproteins (20,21). Since we have previously demonstrated an impairment in triacylglycerol synthesis by stearate (19), it was speculated that stearate treatment would decrease synthesis and secretion of VLDL. Indeed, the rate of VLDL secretion, as measured by [2-^{3}H]glycerol and [^{3}H] or [^{14}C]-labeled fatty acid incorporation into lipids of VLDL recovered in the medium, was the lowest in the stearate-treated cells. These observations coincided with the previous study showing that [1-^{14}C] stearate was incorporated into hepatocyte triacylglycerol at a rate markedly lower than that of labeled myristate, palmitate, or oleate (19). Taken together, these results indicate that an impairment of triacylglycerol synthesis is in part responsible for the depressed VLDL assembly and secretion by hepatocytes treated with stearate.

In addition to the requirements of lipid components, the synthesis of VLDL is dependent on the supply of apoB in the liver (31). Accordingly under the conditions when hepatic VLDL secretion is stimulated, the amount of newly synthesized apoB is increased and so is the amount secreted by the liver (32). Conversely, decreased hepatic secretion of VLDL is accompanied by a reduction of apoB secretion (31). However, it has been demonstrated that dietary fish oil reduces hepatic secretion of VLDL without parallel changes in apoB synthesis (18) and secretion (33). Consistent with this, the

TABLE 2
Effects of Individual Fatty Acids on apoB Content and [^{35}S]Methionine Incorporation into apoB in Cultured Rat Hepatocytes[a]

	Control	Myristate	Palmitate	Stearate	Oleate
		ApoB content (% of control)			
Cellular					
apoB100		92 ± 3	103 ± 6	91 ± 8	106 ± 6
apoB48		96 ± 12	107 ± 7	93 ± 13	109 ± 9
Medium					
apoB100		87 ± 20	93 ± 15	84 ± 25	89 ± 20
apoB48		87 ± 20	96 ± 16	82 ± 24	91 ± 17
% in medium					
apoB100	48.1 ± 5	46.2 ± 4	45.6 ± 5	45.3 ± 4	43.2 ± 1
apoB48	47.2 ± 6	44.6 ± 3	44.5 ± 5	43.3 ± 5	42.6 ± 1
		[^{35}S]Methionine incorporation into apoB (cpm/mg protein/4 h)			
Cellular					
apoB100	222 ± 29[c]	207 ± 16[c]	158 ± 11[a]	174 ± 7.8[b]	171 ± 27[a,b]
apoB48	68 ± 6	69 ± 6	54 ± 1	54 ± 2	53 ± 2
Medium					
apoB100	1055 ± 375	1420 ± 188	1160 ± 419	1238 ± 172	941 ± 287
apoB48	115 ± 6.5	183 ± 63	135 ± 31	160 ± 31	132 ± 40
Total					
apoB100	1277 ± 401	1628 ± 186	1319 ± 431	1416 ± 169	1112 ± 302
apoB48	183 ± 4.2	253 ± 60	189 ± 32	215 ± 29	185 ± 47
total	1460 ± 405	1881 ± 223	1508 ± 448	1631 ± 143	1297 ± 300
% in medium[b]					
apoB100	81.5 ± 4.5	87.1 ± 1.7	87.1 ± 3.4	87.3 ± 1.6	83.9 ± 4.2
apoB48	62.8 ± 3.4	71.4 ± 7.8	70.7 ± 5.3	74.1 ± 4.1	70.7 ± 4.1

[a]Values are means ± SD for four samples. Hepatocytes were incubated with 0.5 mM fatty acids and [^{35}S] methionine for 4 h. Apolipoprotein B (apoB100) and apoB48 were immunoprecipitated and separated by SDS gel electrophoresis. ApoB contents were determined by gel densitometry.
[b]% in medium = medium apoB/(cellular apoB + medium apoB) × 100. Values with different superscript letters in the same row are significantly different at P 0.05.

present study showed that stearate and other fatty acids did not alter cellular contents of apoB100 and apoB48, or the rate of apoB synthesis. Extensive degradation of apoB in the endoplasmic reticulum, however, may limit apoB abundance for synthesis and secretion of VLDL (34,35). Eicosapentaenoic acid and docosahexaenoic acid, for example, have been shown to increase the degradation of the intracellular apoB with a concomitant reduction in VLDL secretion by the cultured rat hepatocytes (18). Although the degradation of apoB was not determined in the present study, the unaltered apoB content and rate of synthesis suggest that stearate and other fatty acids have no effect on degradation. Also, it should be noted that although chylomicrons, VLDL, intermediate density lipoprotein, and LDL are known to contain apoB100 and/or apoB48, an apoB48-containing particle with the density of high density lipoprotein (HDL) has been identified (36). Unlike HDL, apoB48-containing HDL-like particle is destined to VLDL formation by acquiring lipids (36). Oleate has been shown to stimulate the conversion of the HDL-like particle to VLDL (36). It is not clear, however, whether such stimulation could explain the present results demonstrating an increase in VLDL lipid secretion by exogenous fatty acids without increasing apoB48.

The clearance of VLDL from the circulation is an important determinant of plasma VLDL and LDL concentrations (33). The rate of VLDL clearance, on the other hand, is determined by their particle size (15,37). In general, larger VLDL particles are removed more rapidly than smaller counterparts *via* LDL-receptor apoE-specific binding (38,39). Further, larger VLDL particles are enriched with apoE (37,40), and the enrichment of apoE may be responsible for the facilitated removal of large VLDL from the blood through LDL receptor mediated mechanism (41). Although no attempt was made to quantify apoE in the present study, VLDL particles produced by the cultured hepatocytes treated with stearate had a mean particle size which was 1.7 and 2.0 times that of the cells treated with palmitate and myristate, respectively. LDL is derived primarily from VLDL conversion in the circulation (38,42), although direct synthesis of LDL in the liver has been reported (42). It is therefore reasoned that, aside from a reduction in VLDL secretion, the increased VLDL particle size by stearate could further limit the availability of the precursor for LDL synthesis.

The mechanism by which stearate increases VLDL particle size is not readily understood. Large VLDL particles are known to contain more triacylglycerol and less cholesteryl

ester than small VLDL (38,43). Interestingly, VLDL particles of stearate-treated cells contained less both newly synthesized cholesteryl ester and triacylglycerol. It should be pointed out that VLDL particle size is also influenced by the ratio of triacylglycerol to apoB (44). A decrease in the ratio by decreasing lipids and either increasing or maintaining the same apoB content could lead to small VLDL particle size (44). In direct contrast, the present study showed that stearate increased particle size despite a reduction in triacylglycerol secretion and constant apoB100 content. The reason for this unexpected observation is obscure. It is known that the capacity of apoB100 to assemble lipids into VLDL is dependent on length of apoB100 polypeptides. The lipid assembly capacity is the highest by full-length polypeptide of 4536 amino acids and decreases with decreasing the peptide chain (36). Whether stearate inhibits the synthesis of full-size apoB100 and hence reduces lipid content of VLDL is yet to be investigated.

In summary, among saturated fatty acids, stearate has the least stimulatory effect on VLDL secretion. Conversely, stearate increases VLDL particle size more markedly than other fatty acids. These metabolic changes may explain in part the neutral effects of stearate on plasma cholesterol reported by other investigators (1–5).

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Inhibition of Pancreatic Cancer Growth by the Dietary Isoprenoids Farnesol and Geraniol

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ABSTRACT: Fruits and vegetables have protective effects against many human cancers, including pancreatic cancer. Isoprenoids are one class of phytochemicals which have antitumor activity, but little is known about their effects on cancer of the pancreas. We tested the hypothesis that isoprenoids would inhibit the growth of pancreatic tumor cells. Significant (60–90%) inhibition of the anchorage-independent growth of human MIA PaCa2 pancreatic tumor cells was attained with 25 μM farnesol, 25 μM geranylgeraniol, 100 μM perillyl amine, 100 μM geraniol, or 300 μM perillyl alcohol. We then tested the relative *in vivo* antitumor activities of dietary farnesol, geraniol, and perillyl alcohol against transplanted PC-1 hamster pancreatic adenocarcinomas. Syrian Golden hamsters fed geraniol or farnesol at 20 g/kg diet exhibited complete inhibition of PC-1 pancreatic tumor growth. Both farnesol and geraniol were more potent than perillyl alcohol, which inhibited tumor growth by 50% at 40 g/kg diet. Neither body weights nor plasma cholesterol levels of animals consuming isoprenoid diets were significantly different from those of pair-fed controls. Thus, farnesol, geraniol, and perillyl alcohol suppress pancreatic tumor growth without significantly affecting blood cholesterol levels. These dietary isoprenoids warrant further investigation for pancreatic cancer prevention and treatment.

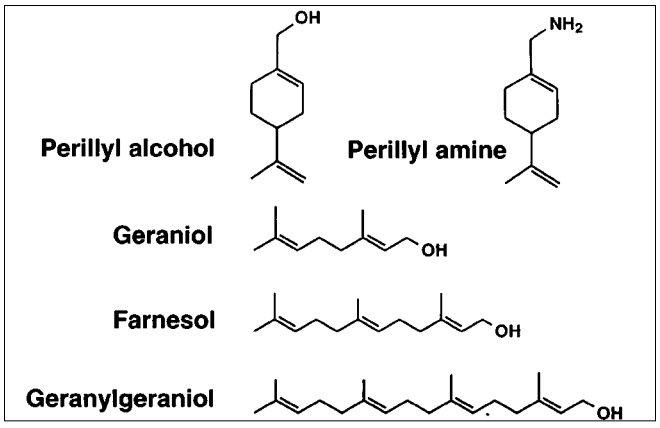
Lipids 32, 151–156 (1997).

Numerous epidemiological studies have revealed that high consumption of fruits, vegetables, and other plant products correlates with a reduction in cancer incidence (1–3). Among the cancers exhibiting reduced incidence in people consuming high levels of plant foods are epithelial cancers of the pancreas (3), colon, breast, and lung (1). Given that these cancers are especially difficult to treat with existing therapeutic modalities, the identification of dietary phytochemicals that have antitumor activity and the investigation of their mechanisms of action may lead to significant advances in the prevention of human cancer by dietary or other means.

Monoterpenes and other isoprenoids are non-nutritive dietary phytochemicals found in the essential oils of citrus fruits, cherry, spearmint, dill, caraway, and other plants. These phytochemicals share the common feature of an isoprenoid structure which is derived from intermediates in the mevalonate pathway. For example, in spearmint and other plants, the monoterpene *d*-limonene is formed by the cyclization of geranylpyrophosphate (4). Limonene then serves as a precursor to other plant monoterpenoids such as perillyl alcohol (Scheme 1). Geraniol, farnesol, and geranylgeraniol (Scheme 1) are the alcohol metabolites of the corresponding pyrophosphates (5,6) which are key intermediates in the mevalonate pathway of both plants and mammals (7). Some specific dietary sources of isoprenoids include perillyl alcohol in cherry and spearmint; *d*-limonene in orange peel oil, caraway, and dill; and geraniol and farnesol in lemongrass.

Some dietary isoprenoids are known to have antitumor activity (8). Limonene has antitumor activity against rodent mammary, liver, lung, stomach, and skin cancers (9–14). Similarly, perillyl alcohol has chemopreventive activity against rat liver cancer (15) as well as chemotherapeutic activity against rodent mammary (16) and pancreatic cancers (17). Geraniol, an acyclic monoterpene, has *in vivo* antitumor activity against murine leukemia, hepatoma, and melanoma cells when administered before and after tumor cell transplantation (18,19). Another dietary isoprenoid, farnesol, has antiproliferative effects on cultured tumor cells (20–22), but its *in vivo* effects on tumor growth are unknown.

Cancer of the pancreas is one of the most deadly of all human cancers, with a 5-year survival rate of only 4% (23).



SCHEME 1

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Abbreviation: NMR, nuclear magnetic resonance.

Patients with pancreatic cancer are usually unresponsive to available chemotherapy. As a result, there is an urgent need for novel, effective treatments for pancreatic cancer. We have recently reported that the dietary monoterpenoid perillyl alcohol has antitumor activity against pancreatic cancer in the hamster (17), but the antitumor effects of other dietary isoprenoids toward pancreatic cancer have not yet been reported. We therefore tested the hypothesis that dietary isoprenoids would inhibit the *in vitro* and *in vivo* growth of pancreatic tumor cells.

MATERIALS AND METHODS

Cell culture. MIA PaCa2 human pancreatic carcinoma cells and PC-1 hamster pancreatic ductal carcinoma cells were cultured as described previously (17,24). All cell lines were maintained in a humidified 37°C, 5% CO_2 incubator and, unless otherwise stated, all tissue culture reagents were purchased from Life Technologies, (Grand Island, NY). All isoprenoids except perillyl amine (see below) were purchased from Aldrich (Milwaukee, WI). Cell proliferation was measured by counting cells on a hemocytometer. To measure anchorage-independent growth, MIA PaCa2 cells were plated in 0.4% agar noble (Difco, Detroit, MI) in DMEM-10% fetal bovine serum over a 0.6% agar noble/DMEM-fetal bovine serum base. Fresh control or isoprenoid medium was added daily for 19 d, at which time colonies of more than 50 cells were scored.

Perillyl amine synthesis. Perillyl amine was synthesized from *N*-perillyl phthalimide as follows. Diisopropylazodicarboxylate (168 μL, 0.85 mmol) was added dropwise in the dark to a solution of perillyl alcohol (100 mg, 0.66 mmol), phthalimide (126 mg, 0.85 mmol), and triphenylphosphine (223 mg, 0.85 mmol) in dry tetrahydrofuran (5 mL) at 23°C. After the solution had been stirring for 4 h, water (20 mL) was added and the aqueous phase was extracted with hexane (3 × 15 mL). The combined organics were washed with brine, dried over $MgSO_4$, and filtered. The filtrate was evaporated *in vacuo* and the resulting residue applied to a 2 × 13 cm silica gel column. Flash chromatography using 1% ethyl acetate/hexane provided *N*-perillyl phthalimide (181 mg, 98%) as a white solid: ^{1}H nuclear magnetic resonance (NMR) δ 7.8, 7.7 (two *m*, 4 H, aromatic), 5.63 (*br s*, 1H, C=CH), [4.70, 4.66 (two *br s*, 2H, C=CH_2)] (*d*, 2H, C=CH_2), 4.18 (*s*, 2H, CH_2N), 2.12 (*m*, 5H, C=CCH_2 and CH), 1.8, 1.4 (*m*, 2H, CH_2), 1.69 (*s*, 3H, CH_3); ^{13}C NMR δ 168.1, 149.6, 133.8, 132.1, 131.9, 123.7, 123.2, 108.7, 43.3, 40.7, 30.3, 27.3, 27.1, 20.6.

N-Perillyl phthalimide (130 mg, 0.46 mmol) was dissolved in 0.3 M methanolic hydrazine hydrate (5 mL). After the solution had stirred for 0.5 h at 23°C, 5% HCl (2 mL) was added and the reaction was stirred for an additional 12 h. The resulting suspension was filtered, and the filtrate was diluted with an equal amount of water, acidified (pH <2) and washed with ether. The organic layer was discarded, and the aqueous layer was basified with solid KOH (pH >10) then extracted twice with ether. The combined ethereal layers were washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. An analytical sample was prepared by passing the residue through a silica gel plug using 3% methyl alcohol/CH_2Cl_2 as an eluent to give pure perillyl amine (62 mg, 88%): ^{1}H NMR δ 5.56 (*br s*, 1H, C=CH), 4.69 (*br s*, 2H, C=CH_2), 3.15 (*br s*, 2H, CH_2N), 2.0 (*m*, 5H, C=CCH_2 and CH), 1.9, 1.5 (*m*, 2H, CH_2), 1.72 (*s*, 3H, CH_3), 1.59 (*br s*, 2H, NH_2); ^{13}C NMR δ 149.9, 138.8, 119.9, 108.5, 48.0, 41.3, 30.5, 27.7, 27.0, 20.7.

In vivo *antitumor activity.* Male Syrian golden hamsters, Eppley colony (Sasco, Omaha, NE), 8–9 wk of age at the time of arrival, were housed in a room with a 12-h light/dark cycle and controlled temperature and humidity. Hamsters were given free access to water and food (meal form Harlan-Teklad rodent diet, Madison, WI) unless otherwise indicated. After a 7–14 d adaptation period, 10-wk-old hamsters were injected s.c. with 4×10^6 PC-1 hamster pancreatic ductal adenocarcinoma cells (passage 17) as described by Egami *et al.* (25). PC-1 cells of passages later than 20 were less capable of forming tumors (data not shown). The s.c. implantation of tumors enabled tumor size to be monitored at multiple timepoints, as described below. In experiment 1, one week prior to PC-1 cell transplantation, 15 hamsters per diet group were randomly assigned to either the control diet or an isoprenoid diet containing 40 g/kg diet perillyl alcohol, 20 g/kg diet farnesol, or 20 g/kg diet geraniol, and these diets were continued for the remainder of the experiment. These were the maximal isoprenoid doses that allowed the hamsters to at least maintain their body weight (data not shown). In experiment 2, 36 hamsters were injected s.c. with PC-1 cells as described above, and, at the time of appearance of a tumor >2 mm in diameter, hamsters were randomly assigned to either the control diet or an isoprenoid diet containing 20 g/kg diet farnesol or 20 g/kg diet geraniol. In both experiments, the isoprenoids were mixed with the meal form diet in a food processor.

All diets were stored at −20°C, and fresh diet was given every day to minimize evaporation of the isoprenoids. Hamster body weight was measured twice weekly, and food intake was recorded daily. Because the animals did not spill significant amounts of food, food intake was measured by weighing the hanging feeder and food before and after the hamster was allowed to eat for 24 h. Control animals were pair-fed to the farnesol animals on a daily basis by providing them with a quantity of food equal to that consumed by the farnesol group on the day before. Food intake among the isoprenoid groups was not different (data not shown). Hamsters were palpated twice weekly, and tumor diameter was measured with calipers. When tumors grew to >20 mm in diameter, or by four weeks on therapy, hamsters were killed by CO_2 asphyxiation, and tumors were removed and measured. All animal procedures were carried out in accordance with institutional approval.

Plasma cholesterol analyses. Plasma was isolated from blood samples obtained at necropsy from the control and isoprenoid-fed animals from experiment 1 and stored at −20°C prior to total cholesterol analyses. Briefly, 1 mL of choles-

terol reagent (Sigma Diagnostics, St. Louis, MO) containing cholesterol esterase, cholesterol oxidase, and peroxidase was added to 10 µL of plasma or a standard solution containing 100–400 mg/dL total cholesterol (Sigma). The samples were incubated at 37°C for 5 min, and the A_{500} was measured within 30 min. A standard curve of A_{500} vs. cholesterol concentration was plotted, and sample total cholesterol concentrations were calculated from the slope of the standard curve.

Statistical analyses. Student's *t*-test and chi-square analyses were carried out with StatisticaMac software, version 4.1, (StatSoft, Inc., Tulsa, OK).

RESULTS

The effects of a number of dietary isoprenoids were first tested on cultured human MIA PaCa2 pancreatic ductal adenocarcinoma cells. We have previously reported that perillyl alcohol inhibits the growth of these cells with an IC_{50} of 290 µM (17). Here, the relative effects of other isoprenoids on PaCa2 cell growth were tested. First, dose-response cell proliferation studies were conducted with 0–400 µM of each isoprenoid. All isoprenoids tested readily inhibited PaCa2 cell growth (Fig. 1). The IC_{50} values for the isoprenoids, determined by extrapolation from the graph depicted in Figure 1, were: geranylgeraniol 25 µM; farnesol 39 µM; perillyl amine 45 µM; and geraniol 265 µM. Then, the effects of these isoprenoids on the anchorage-independent growth of the MIA PaCa2 cells were determined. As shown in Fig. 2, all isoprenoids tested inhibited the growth of the human pancreatic tumor cells. Significant (60–90%) inhibition of human pancreatic tumor cell soft agar growth was attained with 25 µM farnesol, 25 µM geranylgeraniol, 100 µM perillyl amine, 100 µM geraniol, or 300 µM perillyl alcohol ($P < 0.05$ vs. control by Student's *t*-test for each isoprenoid at the indicated concentrations).

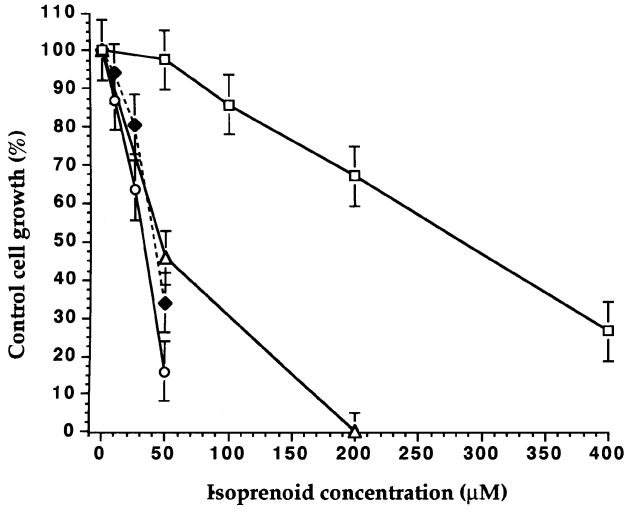

FIG. 1. Inhibition of human pancreatic tumor cell growth by isoprenoids. MIA PaCa2 human pancreatic adenocarcinoma cells were plated at 10,000 per well on day 0 and, beginning on day 1, were treated with 0–400 µM geraniol (◆), farnesol (●), perillyl amine (▲), or geranylgeraniol (■) for 2 d. Cells were harvested and counted on a hemocytometer on days 1 and 3. The cell growth rate, expressed as a percentage of the control, is plotted vs. isoprenoid concentration. The data represent the mean ± SEM, $n = 5$ and are representative of two independent experiments.

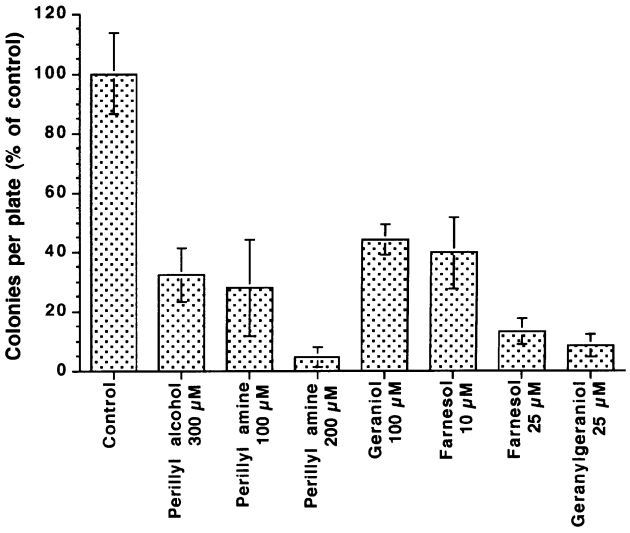

FIG. 2. Suppression of the anchorage-independent growth of human pancreatic tumor cells by isoprenoids. MIA PaCa-2 human pancreatic adenocarcinoma cells were seeded in soft agar at a density of 1000 cells per 60 mm plate, and were incubated in the presence of the indicated isoprenoid for 3 wk. The number of colonies per plate is expressed as a percentage of the control. The data represent the mean ± SEM, $n = 5$ and are representative of two independent experiments.

We then compared the *in vivo* antitumor activity of farnesol, geraniol, and perillyl alcohol in Syrian Golden hamsters bearing transplanted pancreatic tumors (the other isoprenoids were not available in sufficient quantities for animal feeding studies). In experiment 1, hamsters were fed control diet or an isoprenoid diet containing 20 g/kg diet farnesol, 20 g/kg diet geraniol, or 40 g/kg diet perillyl alcohol for 1 wk prior to injection of PC-1 hamster pancreatic ductal adenocarcinoma cells. The animals continued to consume these diets for the remainder of the experiment. The tumor incidence was 5/15 for the control group, 3/15 for the farnesol and geraniol groups, and 4/15 for the perillyl alcohol group. The body weights of the animals consuming the farnesol, geraniol, and perillyl alcohol diets did not differ significantly from one another or from the pair-fed control; group means ranged from 119–127 g at baseline to 119–122 g at the end of the study. Hamsters fed either farnesol or geraniol at 20 g/kg diet exhibited complete inhibition of pancreatic tumor growth ($P < 0.05$ and $P < 0.025$ vs. control, respectively; Fig. 3). Both of these isoprenoids were more potent than perillyl alcohol, which reduced tumor growth by 50% when fed at a dose of 40 g/kg diet (Fig. 3) but did not inhibit tumor growth at a lower dose of 20 g/kg diet (data not shown). Plasma total cholesterol concentrations were then determined for these

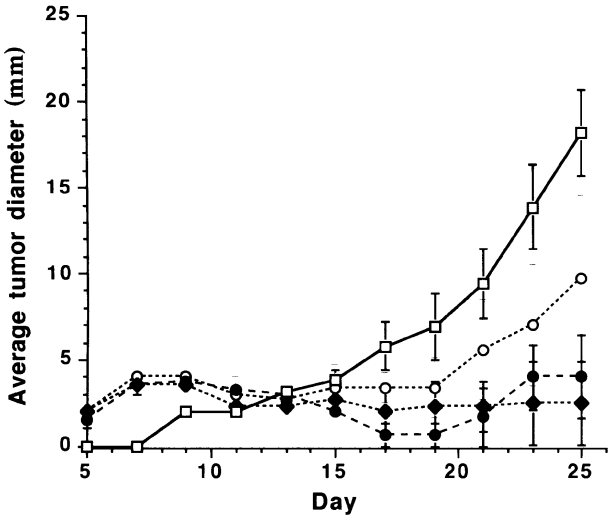

FIG. 3. Inhibition of hamster pancreatic tumor growth by dietary isoprenoids. Hamsters were allowed to acclimate to the control or isoprenoid diets for one week prior to the s.c. injection of PC-1 tumor cells. Average tumor diameter is plotted vs. days after tumor cell injection for animals fed the control diet (❑), 40 g/kg diet perillyl alcohol (○), 20 g/kg diet geraniol (◆), or 20 g/kg diet farnesol (●). The data represent the mean ± SEM, $n = 3$. On day 25, the average tumor diameters of the farnesol and geraniol groups were lower than that of the control ($P < 0.05$ and 0.025, respectively, by Student's *t*-test).

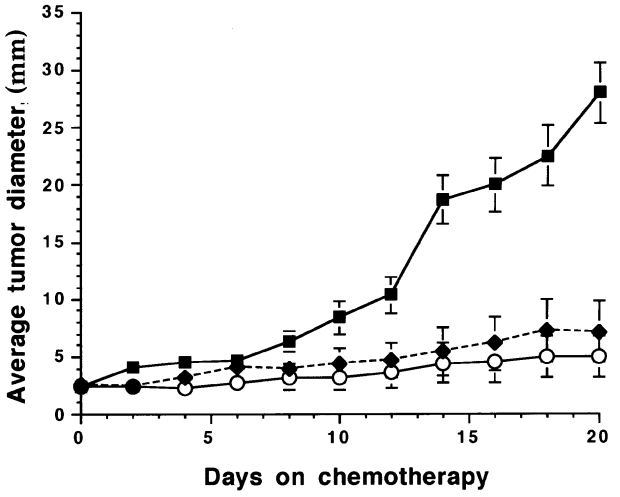

FIG. 4. Chemotherapeutic activity of dietary isoprenoids against pancreatic cancer in the hamster. Animals were randomly assigned to the control (❑), 20 g/kg diet geraniol (◆), or 20 g/kg diet farnesol (○) groups after the detection of a tumor. Average tumor diameter is plotted vs. days on chemotherapy. The data represent the mean ± SEM, $n = 8$. On day 20, the tumor sizes of the farnesol and geraniol animals were significantly less than that of the controls ($P < 0.05$ by Student's *t*-test).

hamsters after 32 d on the respective diets (i.e., 25 d after tumor cell injection). The total plasma cholesterol levels of all hamsters fed farnesol, geraniol, or perillyl alcohol diets were not significantly different from those of the pair-fed control hamsters; group means at the end of the experiment were 135 ± 7; 154 ± 10; 164 ± 11; and 154 ± 10. Similarly, when total plasma cholesterol concentrations of only the tumor-bearing animals were analyzed, there were no statistically significant differences among the diet groups.

Then, in experiment 2, the *in vivo* antitumor activities of farnesol and geraniol were tested in a chemotherapy experiment. Hamsters were injected with PC-1 pancreatic tumor cells as above. Then, when tumors became palpable, the tumor-bearing hamsters were assigned to the 20 g/kg diet farnesol, 20 g/kg diet geraniol, or the pair-fed control group for the remainder of the study. Dietary farnesol or geraniol completely inhibited the growth of the pancreatic tumors ($P < 0.05$ vs. control; Fig. 4). As in experiment 1, the body weights of the isoprenoid-fed animals did not differ from those of the pair-fed controls (data not shown).

Some of the tumor-bearing animals consuming isoprenoid diets exhibited tumor regression, i.e., a tumor that was nonpalpable for three weeks or more. Table 1 contains tumor regression data pooled from experiments 1 and 2. Farnesol and geraniol both caused 8/19 tumors to regress, whereas none of the control tumors regressed. One of the three tumors treated with perillyl alcohol regressed in experiment 1, consistent with our previous observations that 5/31 hamster pancreatic tumors treated with perillyl alcohol regressed (17).

TABLE 1
Isoprenoid-Induced Pancreatic Tumor Regressions

Diet	Tumor regressions[a]	No regression
Control	0	8
Geraniol	8[b]	11
Farnesol	8[b]	11

[a]Tumor regression data are summarized from experiments 1 and 2. Complete tumor regression was scored when tumors were unpalpable for a minimum of three consecutive weeks. Complete tumor regressions were confirmed at necropsy.
[b]Chi-square = 4.79, $P < 0.029$ vs. control.

DISCUSSION

Here, we demonstrate that the dietary isoprenoids farnesol and geraniol effectively inhibit *in vivo* growth of pancreatic tumors either when they are given prior to and following tumor cell injection, or when they are administered following tumor appearance in a chemotherapy setting. These results are consistent with the antitumor activity of geraniol against transplanted murine hepatomas, melanomas, and leukemias (18,19). Farnesol is known to inhibit the growth of cultured tumor cells (20–22), but the data presented here demonstrate for the first time that this isoprenoid has *in vivo* antitumor activity. All of the antitumor effects observed in these experiments occurred at dietary doses that did not cause significant toxicity to the animals. Thus, diet-derived isoprenoids hold promise for improvement in human pancreatic cancer prevention and therapy with low toxicity. While the doses used in this study exceed those likely to occur in foodstuffs, these

diet-derived isoprenoids may be useful as diet supplements or pharmacological agents. The observations that perillyl amine and geranylgeraniol have growth suppressive effects on cultured human pancreatic adenocarcinoma cells suggest that other naturally occurring isoprenoids and isoprenoid analogs may also be effective at preventing and treating pancreatic cancer.

Several interesting structure–activity relationships among the isoprenoids are apparent from these studies. Perillyl amine is more potent than perillyl alcohol in the inhibition of pancreatic tumor cell soft agar growth (Fig. 3), since greater inhibition occurs with 200 μM perillyl amine than with 300 μM perillyl alcohol ($P < 0.05$ by Student's *t*-test). The relative effects of the isoprenoids on cultured human pancreatic carcinoma cell proliferation and soft agar growth were similar (Figs. 1,2). Farnesol was at least sevenfold more potent than either geraniol or perillyl alcohol in the inhibition of culture pancreatic tumor cell growth (17; Figs. 1,2). Geraniol was similar in potency to perillyl alcohol in cultured cells, but was more potent than perillyl alcohol *in vivo*, exhibiting antitumor activity equivalent to that of farnesol (Figs. 3,4). The difference in the relative effects of geraniol and the other isoprenoids may be due to differences in their metabolism or pharmacokinetics in cultured cells and *in vivo*. Perillyl alcohol is extensively metabolized *in vivo* (14,26), but less is known about the metabolism and pharmacokinetics of farnesol (27) and geraniol (28) in mammals.

Isoprenoids have multiple effects on mevalonate metabolism, and some of these effects may account for their antitumor activities in some cell types or species (8). The monoterpenes limonene and menthol inhibit hepatic HMG-CoA reductase activity in the rat (29,30) and can reduce serum cholesterol (31). More recently, perillyl alcohol has been shown to inhibit ubiquinone and cholesterol biosynthesis in cultured NIH3T3 murine fibroblast cells (32). In these cells, the inhibition of cholesterol biosynthesis occurred at the conversion of lanosterol to cholesterol, i.e., downstream of HMG-CoA reductase. Farnesol, a metabolite of the mevalonate pathway intermediate farnesylpyrophosphate (5), has been proposed as a negative regulator of HMG-CoA reductase (33,34). In the experiments described here, substantial inhibition of hamster pancreatic tumor growth by farnesol and by geraniol (Figs. 3,4) was attained in the absence of an effect on total plasma cholesterol. Thus, the mechanism by which isoprenoids inhibit pancreatic tumor growth in the Syrian golden hamster is not likely to involve suppression of HMG-CoA reductase activity.

Both farnesol (21) and perillyl alcohol (15) have been reported to induce apoptosis. Farnesol induces cell death in lymphoma, cervical carcinoma, and other cell types (20–22), and this effect is more pronounced in malignant cells than in normal cells. Similarly, perillyl alcohol has been shown to significantly induce apoptosis in chemically-induced rat liver tumors (15). Thus, it is possible that the antitumor activities of perillyl alcohol, farnesol, and other isoprenoids against pancreatic cancer are due to isoprenoid stimulation of apoptosis. In addition, the recent identification of a rat nuclear receptor that is activated by farnesol (35) suggests that farnesol and perhaps other isoprenoids may have transcriptional effects that play a role in their anticancer activities. The testing of this hypothesis will await the identification of farnesol receptor-responsive genes.

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Impaired Cholesterol Esterification in the Plasma in Patients with Breast Cancer

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ABSTRACT: An important factor which determines the movement of cholesterol in and out of the cells is the free cholesterol (FC)/esterified cholesterol (EC) ratio in the plasma. Although this ratio has been shown to be increased in several types of malignancies in humans as well as experimental animals, it is not known whether such an abnormality is found in breast cancer patients. Furthermore, the reasons for such an increase in cancer patients are unknown. We studied the plasma lipid composition and the activity of lecithin–cholesterol acyltransferase (LCAT), the enzyme responsible for the formation of most of EC in human plasma, in 12 women with breast cancer and 9 age-matched control women. The plasma EC concentration was found to be significantly decreased in cancer patients, whereas the FC concentration was unchanged, leading to increased FC/EC ratios ($P < 0.05$). The concentration of phosphatidylcholine, the acyl donor in the LCAT reaction, was decreased significantly, whereas all other phospholipids were unaffected. The cholesterol-esterifying activity of LCAT was significantly lower in cancer patients, whether assayed with endogenous substrates ($P < 0.05$), or with an exogenous substrate ($P < 0.01$). However, another function of the enzyme, namely the lysolecithin acyltransferase activity, was increased ($P < 0.02$), indicating that the enzyme concentration in plasma may not be decreased. These results show that the increase in the FC/EC ratio in cancer patients is due to an impaired esterification of cholesterol by plasma LCAT, probably due to an alteration in the composition of substrate lipoproteins, or the presence of an inhibitory factor.

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Several studies have reported abnormalities in the lipid and lipoprotein composition in various types of malignancies in humans as well as in experimental animals. These abnormalities include hypocholesterolemia (1,2), hypertriglyceridemia (3), and decreased high density lipoproteins (HDL), especially the HDL_2 subfraction (1,3–5). Many of the lipid disorders appear to be reversed following chemotherapy (6), indicating that they may be the result of malignancy itself.

One of the compositional abnormalities found in various types of human malignancies is an increased ratio of free cholesterol (FC)/esterified cholesterol (EC) (7). When mice were transplanted with ascites tumor, the ratio of FC/EC in plasma increased 2.5 fold within 7 d, although the total cholesterol decreased significantly (8). A similar increase in this ratio was reported in nude mice with pancreatic acinar cell carcinoma (9). This increase primarily resulted from a decrease in EC concentration, whereas the FC concentration remained unchanged. In contrast to other cancer patients, it is not known whether there is an increase in the FC/EC ratio of breast cancer patients. The original study of Saier *et al.* (7) included only 3 breast cancer patients and the results were inconclusive. Other workers reported an increase in FC but no change in EC in the plasma of breast cancer patients (10). Since FC is required for the growth of all cells and since the FC/EC ratio in the surrounding medium affects the movement of cholesterol in and out of cells (11), it is important to determine the mechanisms by which the FC/EC ratio is increased, and whether it is directly related to tumor growth. The possible causes of the increase are: an increase in the synthesis of FC by tissues and its subsequent secretion into circulation, a decreased clearance of FC from plasma, an accelerated selective uptake of EC, and an impaired esterification of cholesterol in the plasma. Although enhanced cholesterogenesis has been shown to occur in growing tumors (12), it is unlikely that an increase in the synthesis of FC is responsible because the total plasma cholesterol is reduced in most cancer patients. On the other hand, a decreased esterification of plasma cholesterol by lecithin–cholesterol acyltransferase (LCAT) may explain not only the increase in FC/EC ratio, but also the decrease in HDL_2 concentration, because the conversion of HDL_3 to HDL_2 is an LCAT-dependent process (13). Most of the EC in human plasma is formed by the action of LCAT, which transfers a fatty acid from phosphatidylcholine (PC) to FC (14). Only a small percentage of plasma EC is derived from the action of tissue acyl CoA/cholesterol acyltransferase in humans, although this reaction may contribute a significant percentage of plasma EC in other species (15,16). The possible role of decreased LCAT activity in cancer patients has not

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Abbreviations: EC, esterified cholesterol; FC, free cholesterol; FER, fractional esterification rate; HDL, high density lipoproteins; LAT, lysolecithin acyltransferase; LCAT, lecithin–cholesterol acyltransferase; LDL, low density lipoproteins; MER, molar esterification rate; PC, phosphatidylcholine; TG, triacylglycerol; TLC, thin-layer chromatography.

been investigated in detail. Studies in leukemic patients (17) and in mice bearing ascites tumors (8) found no significant alteration in the enzyme activity, whereas a significant reduction was found in mice with pancreatic cancer (9). To our knowledge, no studies have been carried out in breast cancer patients.

The aim of the present study was to investigate whether the FC/EC ratio is increased in breast cancer, and to determine the possible mechanisms for the increase. The results presented here show that the plasma FC/EC ratio is indeed significantly increased in breast cancer patients, and that this increase is most likely due to a decreased LCAT activity.

MATERIALS AND METHODS

Patients. Plasma samples were obtained from 12 women with histologically proven breast carcinoma, and 9 control subjects. One of the 12 patients had bilateral cancer. The mean age was 53 years (range 40–73 years). The pathology included infiltrating ductal carcinoma in 11 tumors, infiltrating lobular carcinoma in one, and ductal carcinoma *in situ* without invasion in one. The average tumor size was 2.4 cm (range 0.8 cm to 5.2 cm). Five of the 12 patients had histologically involved axillary lymph nodes. The estrogen receptor status was positive in 10 tumors, negative in 1, and borderline in 2. The progesterone receptors were positive in 12 and negative in 1. There was no evidence of metastasis in liver or any other distant site. Definitive local regional management included partial mastectomy with axillary dissection and subsequent radiation therapy in 9 patients, modified radical mastectomy with subsequent radiation therapy in 1, modified radical mastectomy alone in 1, and Halsted radical mastectomy with subsequent radiation therapy in 1. Postoperatively, 5 patients received adjuvant cytotoxic chemotherapy, and 6 patients received adjuvant hormonal therapy (tamoxifen). No patient was on chemotherapy, hormone therapy, or radiation therapy at the time of blood drawing. Control subjects were healthy women volunteers (ages 35–65) who had no history of cancer. None of the patients or controls had hyperlipidemia, diabetes, or hepatic or renal disease, and none was on medications which are known to affect plasma lipid concentrations. Informed consent was obtained from all subjects, and the blood samples were drawn in EDTA, following the overnight fasting. Patient and control samples were processed at the same time, under the same conditions.

LCAT assays. LCAT activity was determined by two different procedures. In the proteoliposome method, which employs an exogenous substrate (18), the labeled proteoliposomes containing 4-^{14}C labeled FC (25,000 dpm/reaction; NEN Dupont, Boston, MA) egg PC, and apoprotein A-I at the molar ratios of 12.5:250:0.8, respectively, were prepared by the cholate–dialysis, and aliquots of plasma (20 μL) were incubated with this substrate and 5 mM mercaptoethanol for 60 min at 37°C, in a final volume of 0.4 mL. The reaction was stopped by the addition of 1 mL methanol and the total lipids were extracted (19). FC and EC were separated on silica gel thin-layer chromatography (TLC) plates with the solvent system of petroleum ether/ethyl acetate (85:15, vol/vol), visualized by exposure to iodine vapors, and were identified with the help of authentic standards run on the same plate. The spots corresponding to FC and EC were scraped, and their radioactivity was determined in a liquid scintillation counter following the addition of 5 mL of scintillation fluid (CytoScint; ICN Biomedical, Costa Mesa, CA). The enzyme activity is expressed as the percentage of labeled cholesterol esterified per hour (fractional esterification rate or FER).

In the endogenous substrate assay (20), labeled FC was first equilibrated with endogenous cholesterol in the presence of 2 mM DTNB, a reversible inhibitor of LCAT, at 37°C for 4 h. The inhibition of the enzyme was then released by the addition of 12 mM mercaptoethanol, and the sample was further incubated for 30 min at 37°C. The lipids were extracted (19), and the percentage of labeled cholesterol esterified (FER) was determined as described above. The molar esterification rate (MER) was determined by multiplying the FER with the FC concentration of the plasma.

Lysolecithin acyltransferase (LAT) activity. The LAT activity of LCAT was estimated by the conversion of labeled lyso PC to PC (21). 1-[1-^{14}C]-palmitoyl lyso PC (250,000 dpm/mL; NEN Dupont) was dispersed by vortexing in 10 mM Tris-Cl buffer pH 7.4, at a concentration of 3 mM. An aliquot of this dispersion (100 μL) was incubated with 100 μL of plasma for 2 h at 37°C, in the presence of 5 mM mercaptoethanol, in a final volume of 0.4 mL. The reaction was stopped by the addition of 1 mL methanol, and the total lipids were extracted (19). Lyso PC and PC were separated on TLC plates with the solvent system of chloroform/methanol/water (65:25:4, by vol). The spots corresponding to these two lipids were scraped, and their radioactivity determined in a liquid scintillation counter, after the addition of 0.5 mL water and 5 mL scintillation fluid. The percentage of lyso PC esterified (FER) was calculated from the radioactivity values. The MER was calculated by multiplying the FER with the concentration of lyso PC in the reaction mixture (lyso PC concentration in 100 μL plasma plus 0.3 μmol present in the labeled substrate).

Lipid estimations. FC, total cholesterol, and triacylglycerol (TG) were determined enzymatically using commercial assay kits (21). The EC concentration was derived by subtracting the FC concentration from total cholesterol. For the determination of phospholipids, the total lipids were first extracted from an aliquot of whole plasma, and separated on silica gel TLC plates, with the solvent system of chloroform/methanol/water (65:25:4, by vol). The spots corresponding to lyso PC, sphingomyelin, PC, phosphatidylinositol and phosphatidylserine and phosphatidylethanolamine were scraped, and the lipid phosphorus was determined by the modified Bartlett procedure (22).

Statistics. All differences between the cancer patients and controls were tested for statistical significance by the Student's *t*-test (two-tailed). Correlations between EC concentration and other parameters were analyzed by the Pearson cor-

relation coefficients (SPSS for Windows software; SPSS Inc., Chicago, IL).

RESULTS

Neutral lipid composition of plasma samples from control and cancer subjects is presented in Figure 1. There was no difference in the concentration of FC, but the concentration of EC was significantly lower in breast cancer patients compared to controls. Consequently, the FC/EC ratio was significantly higher in the plasma of cancer patients (Fig. 2). Similar results were previously reported for other types of malignancies (7). Although the TG concentration was higher in cancer plasma, the difference between controls and patients was not statistically significant, because of high variance.

The phospholipid composition of plasma is shown in Figure 3. While there was no statistically significant difference in most phospholipids, the concentration of PC, which is the substrate for LCAT, and is the most abundant plasma phospholipid, was found to be significantly lower in cancer patients, compared to controls. Similar decrease in PC was reported earlier in the HDL subfractions of breast cancer patients (4).

In order to determine whether the increased FC/EC ratio in cancer patients is due to impaired esterification of cholesterol in plasma, the activity of LCAT was assayed by two different methods. In the proteoliposome method, an exogenous labeled substrate was added to the plasma, and the esterification of labeled cholesterol was determined following a 30-min incubation at 37°C. This assay measures the activity of the enzyme without the influence of endogenous substrates and activators, and is therefore believed to reflect the concentration of the active enzyme in the plasma. In the Stokke and Norum procedure (20), labeled cholesterol is first equilibrated with the endogenous substrate before allowing the enzyme reaction to occur. This procedure measures the activity of the enzyme, as influenced by endogenous substrates, activators, and inhibitors, and therefore represents the *in vivo* activity in circulating plasma. In addition to estimating the LCAT activity by these two methods, we also determined the LAT activity of the enzyme, which measures the transfer of an acyl group from PC to lyso PC instead of FC (23). Since this reaction is activated by low density lipoprotein (LDL) instead of apoprotein A-I, this activity also represents the intrinsic activity of the enzyme, without the influence of HDL, which has been shown to be decreased in some cancer patients (1,3–5). However, this activity is affected by the LDL concentration of plasma (24).

Figure 4 shows the various activities of the enzyme, expressed as FER (percentage of substrate esterified per hour). The cholesterol esterification rate, assayed by both exogenous and endogenous substrates, was significantly lower in breast cancer patients. On the other hand, the LAT activity of the enzyme was significantly higher in patients, compared to controls. However, when expressed as molar esterification rate (nmol of substrate esterified/h/mL plasma), the LAT activity was not significantly different in controls and patients (Fig. 5). The cholesterol esterification reaction, in contrast, was significantly lower in patients, even when expressed as MER. Since this value takes into consideration the concentra-

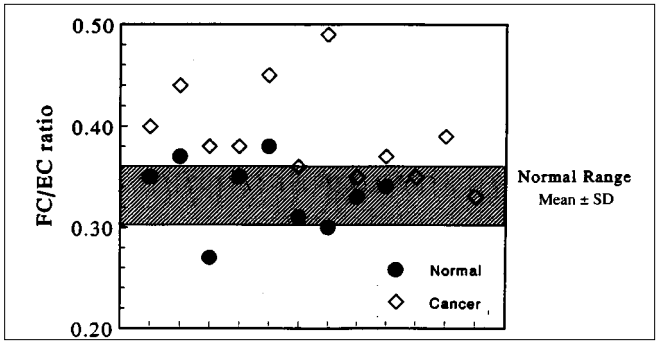

FIG. 2. Individual values of FC/EC ratio in the plasma of patients and controls. The shaded area represents the mean ± 1 SD of normal values. See Figure 1 for abbreviations; ●, normal; ◇, cancer.

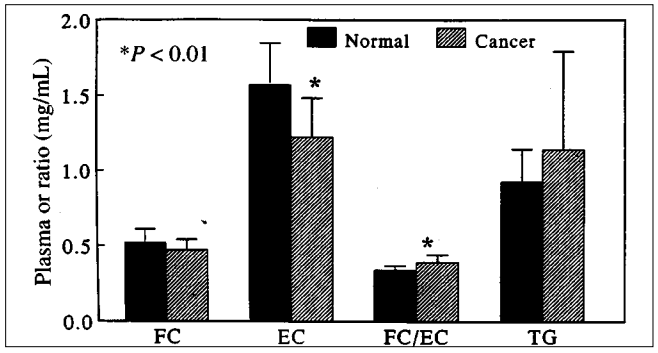

FIG. 1. Neutral lipid composition in plasma of women with breast cancer (n = 12) and in controls (n = 9). Values are expressed as mean ± SD. The significance of difference between the control and cancer samples was determined by Student's t-test. Abbreviations: FC, free cholesterol; EC, esterified cholesterol; TG, triacylglycerol.

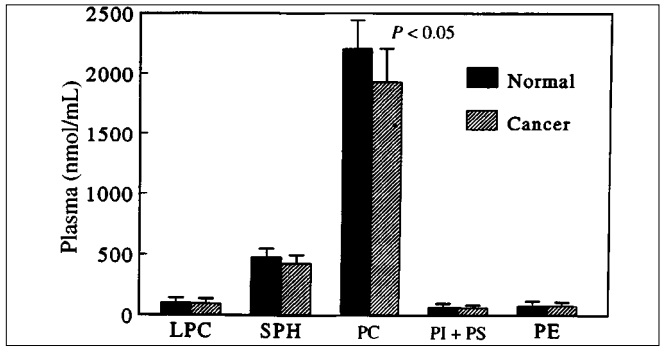

FIG. 3. Plasma phospholipid composition in women with breast cancer (n = 12), and control women (n = 9). Only the differences in PC values were statistically significant. Abbreviations: LPC, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PI + PS, phosphatidylinositol + phosphatidylserine; PE, phosphatidylethanolamine.

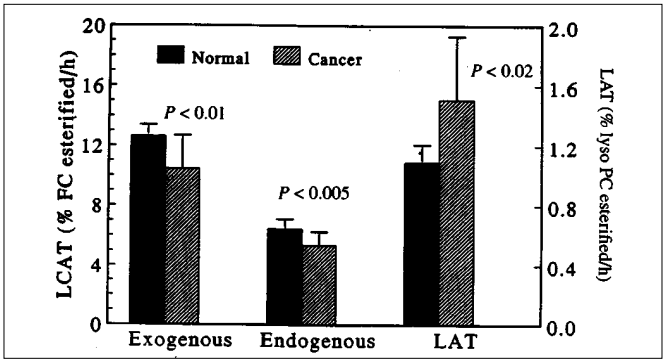

FIG. 4. Fractional esterification rates (FER) of lecithin–cholesterol acyltransferase (LCAT) and lysolecithin acyltransferase (LAT) reactions. LCAT reaction was determined using either an exogenous substrate (proteoliposomes) or endogenous substrates, as described in the text.

tion of endogenous cholesterol, it probably represents the *in vivo* activity more closely. The ratio of cholesterol esterification/lyso PC esterification (LCAT/LAT) was also significantly lower in cancer patients, when expressed as molar activities. Since both activities are carried out by the same enzyme, using endogenous substrates and activators, this decrease in the ratio suggests a specific impairment of cholesterol esterification activity.

Correlations. Since the reason for the increased FC/EC ratio in cancer patients is the decrease in EC rather than an increase in FC, we calculated the correlation of EC concentration with the other parameters measured, in order to determine the possible contributing factors. As expected, the molar LCAT activity (endogenous substrate esterification) showed strong positive correlation with the plasma EC concentration in normal subjects (Table 1). However this correlation was not significant in cancer patients. This suggests that, in addition to the decreased enzyme activity, other unknown factors may have contributed for the lower EC concentration in cancer patients. The EC concentration correlated positively with

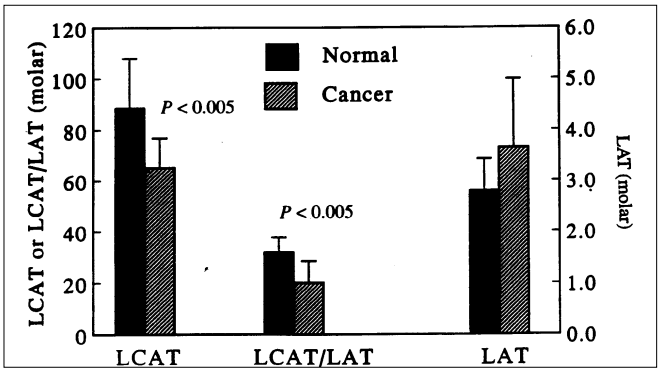

FIG. 5. Molar esterification rates (MER) of LCAT and LAT, and their ratios. The MER for LCAT was calculated by multiplying the FC concentration of plasma with the FER, as obtained in the endogenous substrate assay. The MER of LAT was calculated by multiplying the FER with the concentration of lyso PC present in the plasma and the added labeled substrate. See Figures 1 and 4 for other abbreviations.

TABLE 1
Pearson Correlation Coefficients (*r* values) with EC Concentration[a]

Parameter	Normal (n = 9)	Breast cancer (n = 12)
LCAT, FER (endogenous)	0.19	–0.30
LAT, FER	0.68*	0.02
LCAT, MER	0.77*	0.46
LAT, MER (endogenous)	0.60	0.26
FC	0.80*	0.87**
TG	0.006	0.045
LPC	0.39	0.49
Sphingomyelin	–0.08	0.51
PC	–0.22	0.61*
PI + PS	0.73*	0.52
PE	–0.74*	0.20
Total phospholipids	–0.33	0.64*
FC/PC	0.73*	0.24

[a]EC, esterified cholesterol; LCAT, lecithin–cholesterol acyltransferase; FER, fractional esterification rate; LAT, lysolecithin acyltransferase; MER, molar esterification rate; FC, free cholesterol; TG, triacylglycerol; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine. *$P < 0.05$; **$P < 0.01$.

FC concentration in both the controls and the patients. It also correlated positively with PC, as well as total phospholipids in cancer patients, but not in controls. FC/PC ratio correlated with the EC concentration in both control and cancer subjects, but the correlation was significant only in the former. It was also of interest to note that the LCAT molar activity correlated positively with LAT activity (both FER and MER) in normal subjects, but negatively in cancer patients (results not shown).

DISCUSSION

Cholesterol is an essential membrane component of all mammalian cells, and although all the cells in the body have the capacity to synthesize it, most peripheral cells obtain it from the plasma lipoproteins through the receptor-mediated pathways. In human plasma, over 70% of the cholesterol is in esterified form (EC), and most of this EC is formed in the plasma compartment itself, by the action of LCAT (14). Although an abnormal increase in the ratio of FC/EC was reported in the plasma of experimental animals with tumors and in patients with several other types of cancer (7–9), it is not known whether this abnormality exists in breast cancer patients. The lipid metabolism in breast cancer patients appears to be different from that in other types of cancer. Thus while the total cholesterol is generally decreased in most cancer patients, it was reported to be increased in breast cancer patients (10). Similarly, whereas the EC is decreased in lung cancer and hematologic cancer, it was reported to be unchanged in breast cancer (10).

Our studies clearly show a significant increase in the FC/EC ratio of breast cancer plasma. This increase is primarily due to a decrease in the EC concentration, not to an increase in FC. This observation is in contrast to the results of Alexopoulos *et al.* (10), who reported no change in EC but an increase in FC. The reason for this discrepancy is not clear,

but the patient selection, degree of metastasis, and age of patients may all influence the relative concentrations of FC and EC. It should also be noted that in the study of Alexopoulos *et al.* (10) the concentration of FC in breast cancer patients was not actually higher than in other types of cancer or of other control plasma. The "increase" in FC is mainly due to an unusually low concentration of FC in the specific group of control samples used for comparison.

The decrease in plasma EC appears to be directly related to a decrease in the LCAT activity, as determined by two different assay methods. The lower LCAT activity in cancer patients could be due to a reduction in the plasma concentration of the enzyme, its apoprotein activators, or its lipoprotein substrates. The decrease in the activity as assayed by the proteoliposome method, where the substrates and the activator proteins are not limiting, suggests that the concentration of active enzyme in the plasma may indeed be lower. It has been shown that tumor necrosis factor α is increased in some breast cancer patients (3). Since this cytokine has been shown to inhibit the synthesis of LCAT in experimental animals (25), it is possible that the hepatic synthesis of LCAT is reduced in some breast cancer patients. On the other hand, there was a significant increase in the LAT activity of the enzyme in cancer patients, suggesting that the concentration of the enzyme may be normal, but the activator/inhibitor concentrations may be different in control and breast cancer patients. The LAT activity of the enzyme is activated by LDL, whereas the cholesterol esterification reaction occurs mainly on the HDL particles (23). Since the concentration of HDL is decreased in breast cancer (3,4), it is possible that the cholesterol esterification activity is affected by the concentration or composition of the HDL, or both. It should also be pointed that there is significant decrease in the concentration of PC (Fig. 3) (4), the acyl donor for cholesterol esterification. Another possibility is the presence of an inhibitor of LCAT in cancer plasma. For example, it is known there is an increase in the concentration of certain gangliosides in cancer plasma (26). Our preliminary results show that the gangliosides, such as GM_3 and GM_1, are powerful inhibitors of the enzyme (Liu, M., and Subbaiah, P.V., unpublished observations), suggesting that they may also inhibit the enzyme in some cancer patients. However, further studies are needed to confirm this possibility.

The consequence of lower LCAT activity may not only be related to the higher FC/EC ratio in the plasma but also to the lower HDL levels previously reported (1,3–5). Furthermore, an increase in the FC/EC ratio may favor an influx of FC into the cells, similar to the increased FC/PC ratio, thus providing more cholesterol for cell growth and proliferation. The FC/PC ratio in whole plasma was also slightly higher in cancer patients (0.64 ± 0.10 vs. 0.60 ± 0.12) but was not statistically significant. However, it is possible that this ratio is significantly higher in certain lipoprotein subfractions involved in cholesterol efflux. Previous studies (27,28) have shown that an inhibition of LCAT activity in the serum leads to almost complete inhibition of net cholesterol removal from the cells, and an increase in cellular cholesterol content. A decrease in plasma LCAT activity would therefore lead to an inhibition of the reverse cholesterol transport, and possibly increased tumor growth. It would be of great interest to investigate whether the LCAT activity is restored to normal levels when the tumor is eliminated through surgery and chemotherapy. A recent report (29) showed that the plasma FC is decreased and EC is increased following tamoxifen treatment of postmenopausal women who had breast cancer, suggesting an increase in LCAT activity, although these authors did not specifically determine the LCAT activity in these patients.

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Effects of Dietary Cholesterol and Triglycerides on Lipid Concentrations in Liver, Plasma, and Bile

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ABSTRACT: Dietary cholesterol (CHL) and triglycerides (TG) can influence plasma, hepatic, and biliary lipid composition, but effects on lipids in these three compartments during the early stages of CHL gallstone formation have not been studied in parallel. We fed prairie dogs diets containing one of four test oils (safflower, coconut, olive, or menhaden) at either 5 or 40% of calories, in the presence of 0 or 0.34% CHL, for 3 wk. In the absence of dietary CHL, increases in dietary TG produced 50–200% increases in the concentrations of biliary CHL and hepatic cholesteryl ester (CE), while the concentrations of hepatic free CHL (FC) as well as plasma FC and CE remained relatively unchanged. Increasing dietary CHL to 0.34% resulted in increases in hepatic FC of approximately 50% for all four fats regardless of whether they were supplied at 5 or 40% of calories. CHL supplementation caused more pronounced increases in biliary CHL (200–400%), hepatic CE (50–200%), plasma FC (up to 100%), and plasma CE (up to 150%), and these increases were exacerbated by concurrent supplementation of dietary fat and CHL (biliary CHL: 300–700%; hepatic CE: 100–250%; plasma FC: up to 165%; plasma CE: 100–350%). These results indicate that enhanced secretion of biliary CHL and, to a lesser extent, increased synthesis of hepatic CE, may be primary mechanisms for maintaining the hepatic FC pool. Furthermore, dietary CHL and high levels of fat intake are independent risk factors for increasing biliary CHL concentrations, and adverse effects on lipid concentrations in plasma and bile tend to be exacerbated by ingestion of diets rich in both fat and CHL.

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Diet exerts an important influence over lipid homeostasis, and perturbations in lipid metabolism are important contributing factors in the etiology of diseases such as atherosclerosis and cholesterol (CHL) cholelithiasis. It is now almost universally accepted that hypercholesterolemia is an important independent risk factor for atherosclerosis (1–3), and a number of reports indicate that high levels of dietary CHL (4–7) or saturated fat (8–10) lead to an elevated concentration of plasma CHL carried in the atherogenic low density lipoprotein (LDL) fraction. In addition, high levels of dietary CHL and saturated fat have been linked to secretion of bile supersaturated with CHL (11,12), which is a pre-condition for the development of CHL gallstones (13,14). Diet also has an impact on the levels of plasma triglycerides (TG), and hypertriglyceridemia may be relevant to both atherosclerosis (15) and CHL gallstone disease (16).

It is now widely held that the population should be advised to reduce dietary CHL and saturated fat, but controversy exists over the optimal levels of total fat intake as well as which dietary TG represents the optimal substitute for saturated fats. Evidence suggests that n-6 polyunsaturated fats are effective in lowering plasma CHL concentrations, with most of the reduction occurring in the atherogenic LDL fraction (17,18). However, a potential drawback to the use of these dietary fats is a reported decrease in the antiatherogenic high density lipoprotein (HDL) fraction (19,20). Monounsaturated fats also reduce levels of plasma CHL (17–20), with the added advantage that the reduction appears to be specific for the LDL fraction (19,20). Finally, the n-3 polyunsaturated fats derived from marine sources appear to be highly effective in the treatment of hypertriglyceridemia (21–23), but they have been reported to both decrease (22–24) and increase (25,26) plasma CHL concentrations.

One potential problem with recommending consumption of oils which lower plasma lipoproteins is they may be associated with increased biliary secretion of CHL. A group of men ingesting a hypocholesterolemic diet enriched in n-6 polyunsaturated vegetable oils demonstrated an increased incidence of CHL gallstones (27). A recent study in prairie dogs also suggested that consumption of n-6 TG might increase the biliary secretion of CHL (28). However, the effect of dietary TG on biliary lipid secretion has not been systematically examined.

The present studies were undertaken to examine lipid metabolism during the early stages of CHL gallstone formation in the prairie dog, a commonly used animal model for CHL cholelithiasis (29,30). The prairie dog is a particularly good model for these studies, since similarities with humans have been demonstrated for both plasma (31) and biliary lipids (32). By analyzing the effects of varying levels of dietary CHL as well as varying types and levels of dietary fat on he-

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Abbreviations: BS, bile salt; CE, cholesteryl ester; CHL, cholesterol; CSI, cholesterol saturation index; FC, free cholesterol; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; PL, phospholipid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TG, triglyceride; VLDL, very low density lipoprotein.

patic, plasma, and biliary lipids in the prairie dog, these experiments examined the role of diet in lipid homeostasis.

MATERIALS AND METHODS

Animal studies. Animal studies were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine. Adult female prairie dogs *(Cynomys ludovicianus)* weighing 0.8–1.2 kg were purchased from R-Zoo (Neshkoro, WI). During an initial 1 wk equilibration period, all animals received a standard rodent chow diet (Purina, Purina Mills, Inc., St. Louis, MO). Six animals were then assigned to each of 16 diets for 3 wk as follows: (i) four diets containing trace CHL and 5% of calories from one of the following test oils: safflower, coconut, olive, or menhaden oil; (ii) four diets containing trace CHL and 40% of calories from test oil; (iii) four diets containing 0.34% CHL and 5% of calories from test oil; or (iv) four diets containing 0.34% CHL and 40% of calories from test oil. All diets contained at least 1 g of safflower oil/kg diet to prevent essential fatty acid deficiency. Tables 1 and 2 provide a detailed description of the diets. All diets appeared to be equally well accepted by the animals.

Three weeks after assignment to one of the 16 dietary groups, animals were fasted overnight and anesthetized with ketamine HCl 100 mg (Ketalar; Parke-Davis, Division of Warner-Lambert Co., Morris Plains, NJ) and acepromazine maleate 2 mg (PromeAce; Fort Dodge Laboratories, Fort Dodge, IA), delivered together as a single intramuscular injection. The gallbladder was removed, and its contents were aspirated for analysis of biliary lipids. A 1 g sample of liver was harvested from the inferior edge of the right lobe for measurement of hepatic lipids. Blood was collected from the inferior vena cava for measurement of plasma lipids, and a fatal dose of anesthetic was administered.

TABLE 1
Composition of Prairie Dog Diets

	Group			
	A	B	C	D
Dietary cholesterol	trace	trace	0.34%	0.34%
Dietary triglyceride:	5%	40%	5%	40%
Cholesterol (g/kg)	—	—	3.4	3.4
Safflower oil (g/kg)	1.0	1.0	1.0	1.0
Test oil (g/kg)	15.2	202.0	15.2	202.0
Soy assay protein (g/kg)	202.3	134.0	202.3	134.0
Sucrose (g/kg)	565.5	486.0	562.1	482.6
Corn starch (g/kg)	139.0	100.0	139.0	100.0
Cellulose (g/kg)	26.0	26.0	26.0	26.0
Mineral mix, Phillips-Hart (g/kg)	40.0	40.0	40.0	40.0
Vitamin mix, Teklad (g/kg)	10.0	10.0	10.0	10.0
Flavor, Sessalom (g/kg)	1.0	1.0	1.0	1.0
Calories from protein (%)	19.0	10.0	19.0	10.0
Calories from carbohydrate (%)	75.7	50.0	75.7	50.0
Calories from fat (%)	5.3	40.0	5.3	40.0

TABLE 2
% Fatty Acid Composition of Prairie Dog Diets

	Test oil							
	Safflower		Coconut		Olive		Menhaden	
Fatty acid	5%	40%	5%	40%	5%	40%	5%	40%
10:0	0.0	0.0	1.2	5.2	0.0	0.0	0.0	0.0
12:0	0.0	0.0	31.4	48.6	0.0	0.0	1.8	1.0
14:0	0.2	0.1	14.6	19.0	0.0	0.0	5.9	8.8
16:0	10.6	7.1	14.3	9.9	16.0	14.6	19.6	21.8
16:1	0.2	0.2	0.0	0.0	1.2	1.4	8.6	12.9
18:0	3.0	2.4	3.8	2.8	2.9	2.3	3.6	3.4
18:1	15.4	15.9	10.8	8.0	49.0	61.8	14.4	13.8
18:2	66.5	72.4	18.8	3.1	27.0	17.5	17.7	2.6
18:3	1.2	0.1	1.4	0.1	1.8	1.0	2.0	0.8
20:4n-6	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.6
20:4n-3	0.0	0.0	0.0	0.0	0.0	0.0	0.8	1.4
20:5n-3	0.1	0.1	0.0	0.0	0.0	0.0	7.7	12.4
22:5n-3	0.0	0.0	0.0	0.0	0.0	0.0	1.2	2.1
22:6n-3	0.0	0.0	0.0	0.0	0.0	0.0	5.5	10.2
Total:	97.2	98.3	96.3	96.7	97.9	98.6	89.2	91.8

Lipid assays. Samples of gallbladder bile were stored at –20°C prior to analysis of total bile salts (BS) by the hydroxysteroid dehydrogenase method of Talalay (33) as modified by Admirand and Small (34). The sample of liver was homogenized in methanol immediately after harvesting, and lipids were extracted from fresh aliquots of bile, plasma, and liver homogenate by the method of Folch *et al.* (35). Hepatic, plasma, and biliary phospholipids (PL) were measured by the method of Bartlett (36). Biliary CHL was measured by the method of Rudel and Morris (37). The CHL saturation index (CSI) was calculated as described by Kuroki *et al.* (38). Plasma total CHL was saponified in ethanolic KOH, and plasma total and free CHL (FC) were measured by the CHL oxidase method (39). Plasma cholesteryl ester (CE) was determined as the difference between total and free CHL. Hepatic FC and CE were separated by thin-layer chromatography on silica gel G plates in the solvent system hexane/diethyl ether/acetic acid (70:30:1) and eluted with chloroform/methanol/diethyl ether (1:1:1). After saponification of the CE fraction in ethanolic KOH, hepatic FC and CE were measured by the CHL oxidase method (39). Hepatic and plasma TG were measured using a commercially available kit (Sigma Chemical Co., St. Louis, MO).

Analysis of bile salt species by HPLC. The relative proportions of individual BS species in gallbladder bile were measured as described by Rossi *et al.* (40). High-performance liquid chromatography (HPLC) was performed with a Shimadzu (Columbia, MD) system consisting of an LC-6A solvent delivery system, an SPD-6A variable wavelength detector, and a C-R3A Chromatopac integrator; elution was performed with an Ultrasphere ODS 4.6 × 250 mm reverse-phase column (Beckman Instruments, Fullerton, CA).

Data analysis. Results for groups of animals (n = 4–6) are reported as the mean ± SEM. All tests of significance were conducted using Statistical Analysis Software (SAS) licensed to Boston University. Differences among the 16 dietary

groups were tested for statistical significance by analysis of variance using the General Linear Models procedure. When analysis of variance indicated significant differences among the groups, *post hoc* multiple comparisons were conducted with Least Squares Means. Relationships between dietary parameters (type of oil, % fat, and % CHL) and hepatic, plasma, and biliary lipids also were examined with three-factor analysis of variance using the General Linear Models procedure.

RESULTS

Free cholesterol concentrations. Changes in the levels of dietary TG and CHL influenced FC concentrations in liver, plasma, and bile, but the pattern of change differed among these three compartments. Not surprisingly, the lowest levels of FC were seen with diets which did not contain CHL. Figure 1 (A and B) shows that supplementation with olive, safflower, or menhaden oil in the absence of dietary CHL resulted in similar low concentrations of FC in liver and plasma. Coconut oil tended to give slightly higher values for both hepatic and plasma FC. Increasing dietary TG from 5 to 40% of calories had no influence on the levels of FC in either liver ($P = 0.30$) or plasma ($P = 0.16$), regardless of the type of dietary TG. A different pattern of response was seen in bile, however, in which FC concentrations were low for all four dietary TG when provided at 5% of calories, but increased by

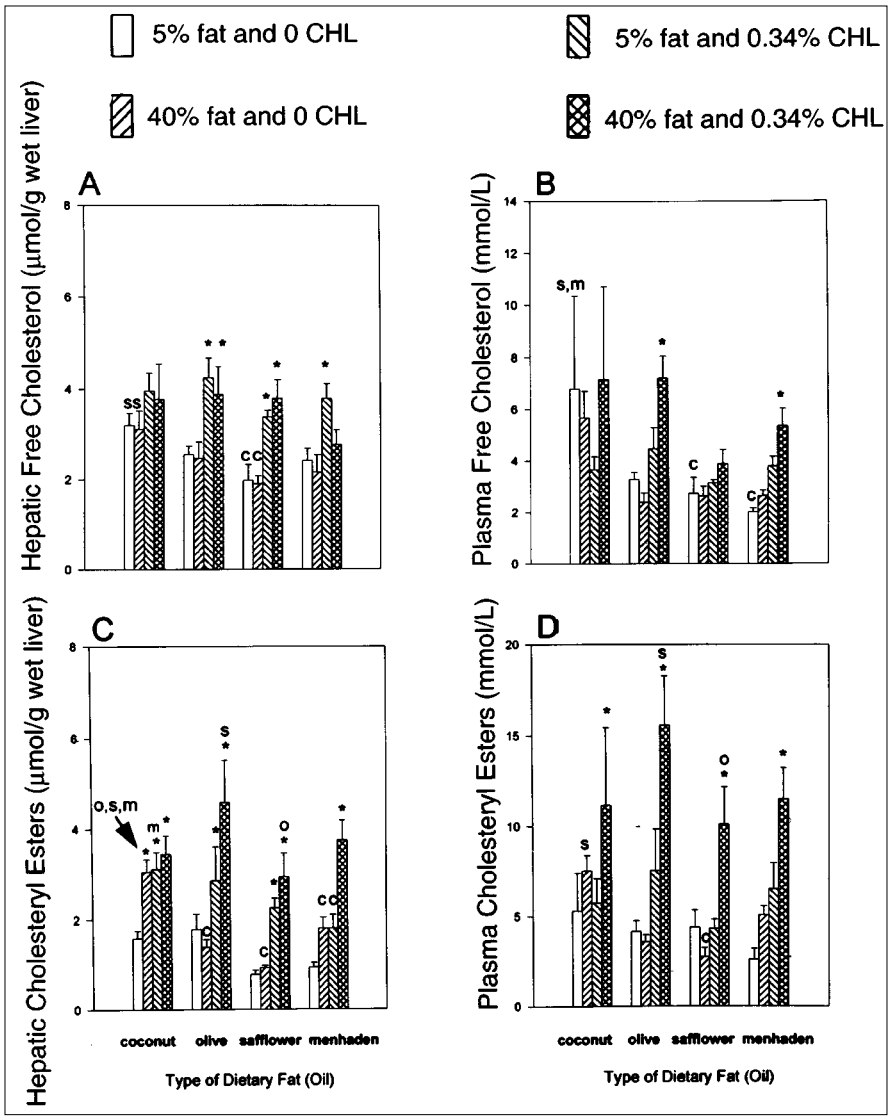

FIG. 1. Effect of dietary fat and cholesterol on the concentrations of cholesterol (CHL) in liver and plasma. A: Hepatic free cholesterol. B: Plasma free cholesterol. C: Hepatic cholesteryl ester. D: Plasma cholesteryl ester; c: $P < 0.05$ compared to coconut oil; o: $P < 0.05$ compared to olive oil; s: $P < 0.05$ compared to safflower oil; m: $P < 0.05$ compared to menhaden oil; *$P < 0.05$ compared to 5% fat, no CHL group within same dietary oil.

50–200% when dietary fat caloric intake was increased to 40% ($P = 0.0001$) (Fig. 4A).

As shown in Figures 1B and 3A, dietary supplementation with CHL increased the concentrations of FC in plasma by up to twofold ($P = 0.04$) and in bile by 200–400% ($P = 0.0001$). Furthermore, in these two compartments, there was a trend for the CHL-induced increases in FC to be exacerbated by concurrent high levels of dietary TG. With the exception of biliary CHL in the menhaden oil group, this additive effect was observed with all four types of dietary fat. The concentration of FC in plasma increased by up to 165% in response to supplementation with both fat and CHL ($P = 0.06$), with safflower oil having the least detrimental effect. The changes were even more dramatic in bile, where the concentration of FC increased by 300–700% for all types of oil but menhaden. In contrast to the results obtained in plasma and bile, Figure 1A shows that the inclusion of 0.34% CHL in the diets consistently caused hepatic FC levels to rise, but regardless of the type or amount of dietary TG, hepatic FC only increased by approximately 50% to 3.5–4.0 µmol/g liver. In the case of hepatic FC, there was no additive effect of dietary fat and CHL ($P = 0.67$).

Cholesteryl ester concentrations. As illustrated in Figure 1C, hepatic concentrations of CE were similar among the four dietary oils when provided at 5% of calories without dietary CHL. Hepatic CE increased in response to dietary CHL ($P = 0.0001$) and increases in fat ($P = 0.0001$), but the effect varied depending on the type of dietary TG. With coconut oil or menhaden oil, hepatic CE rose in response to both dietary CHL (coconut: 140%; menhaden: 60%) and an increase in dietary fat (coconut: 140%; menhaden: 60%). In contrast, with olive oil or safflower oil, increases in dietary fat alone had no apparent effect on CE, but supplementation of dietary CHL caused significant increases in CE (olive: 60%; safflower: 200%). Despite these apparent differences, there was a trend in each of the four oil groups for the greatest elevations in hepatic CE (100–250%) to occur when diets were enriched with both fat and CHL ($P = 0.16$).

Figure 1D shows a somewhat similar pattern of response in plasma CE. Increases in dietary fat had a variable influence ($P = 0.0004$), with modest increases in plasma CE occurring only in response to increases in coconut oil or menhaden oil. Dietary CHL alone also had a variable effect ($P = 0.0001$), with 80 and 150% increases in plasma CE for olive oil and menhaden oil, respectively. Nevertheless, marked increases of 100–350% were seen with diets containing both CHL and a high level of dietary fat for all four dietary oils ($P = 0.004$).

TG concentrations. Figure 2A shows that when fat intake was limited to 5% of calories, the concentration of hepatic TGs did not differ significantly among the four types of di-

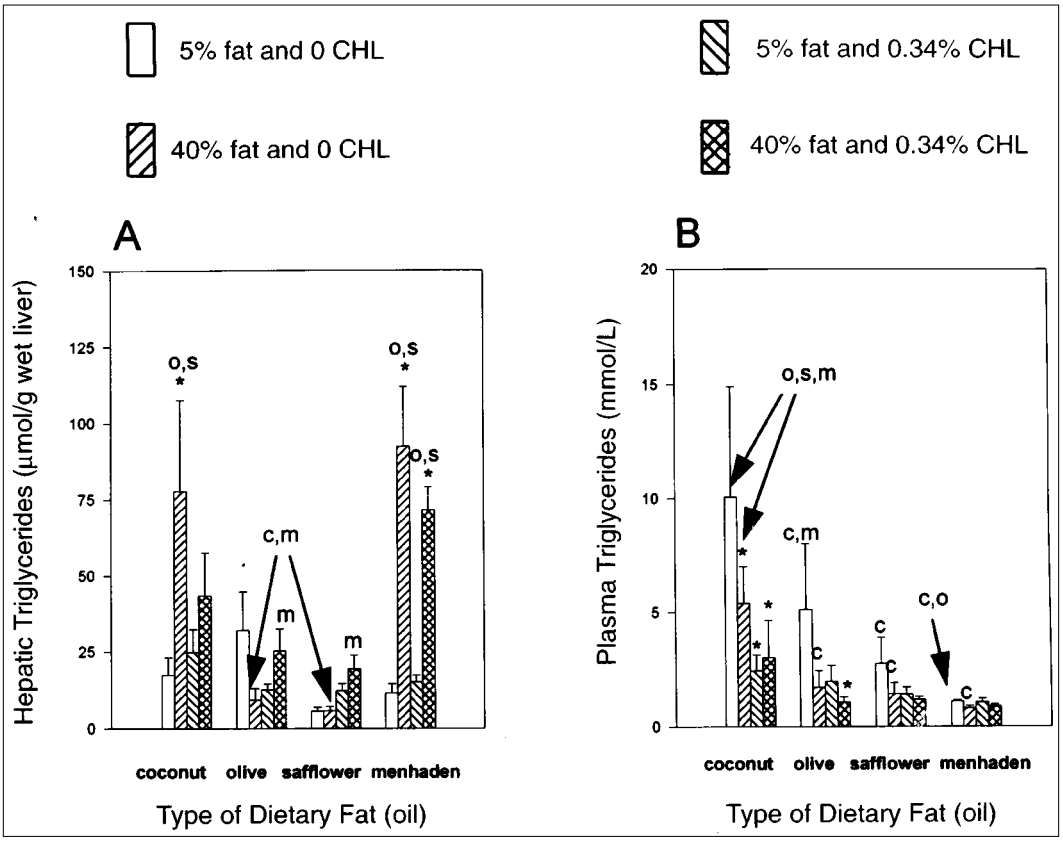

FIG. 2. Effect of dietary fat and cholesterol on the concentrations of triglyceride in liver and plasma. A: Hepatic triglyceride. B: Plasma triglyceride. c, o, s, m, * and abbreviation as described in Figure 1.

etary oils, and hepatic TG concentrations also appeared to be relatively unaffected by dietary supplementation with CHL ($P = 0.71$). On the other hand, there was a variable effect of increased dietary fat among the four dietary oils ($P = 0.0001$). Diets with high levels of coconut oil or menhaden oil produced substantial increases in hepatic TG concentrations, while increases in olive oil or safflower oil did not. The highest concentrations of hepatic TG were seen with the two diets containing 40% of calories from menhaden oil. The increases in TG concentrations in the liver in response to coconut or menhaden oil were not accompanied by similar increases in plasma TG (Fig. 2B). In fact, the menhaden oil diets consistently produced the lowest concentrations of TG in plasma. The highest plasma TG concentrations were seen with 5% coconut oil and 5% olive oil in the absence of dietary cholesterol, but plasma TG levels varied substantially within these two groups.

Hepatic and plasma phospholipid (PL) concentrations. There were few significant changes in the concentration of either hepatic or plasma PL (data not shown).

Biliary lipid concentrations. The effects of dietary fat and CHL on the lipid composition of gallbladder bile are illustrated in Figure 3. As noted above, the FC concentration in gallbladder bile was low for all four dietary TG when provided at 5% of calories, but increased when any of the dietary fats was increased to provide 40% of calories. Biliary FC concentrations increased even more when the diet was supplemented with 0.34% CHL, and, except for menhaden oil, the greatest concentrations in biliary FC occurred when diets contained both CHL and high levels of fat. Changes occurred in biliary

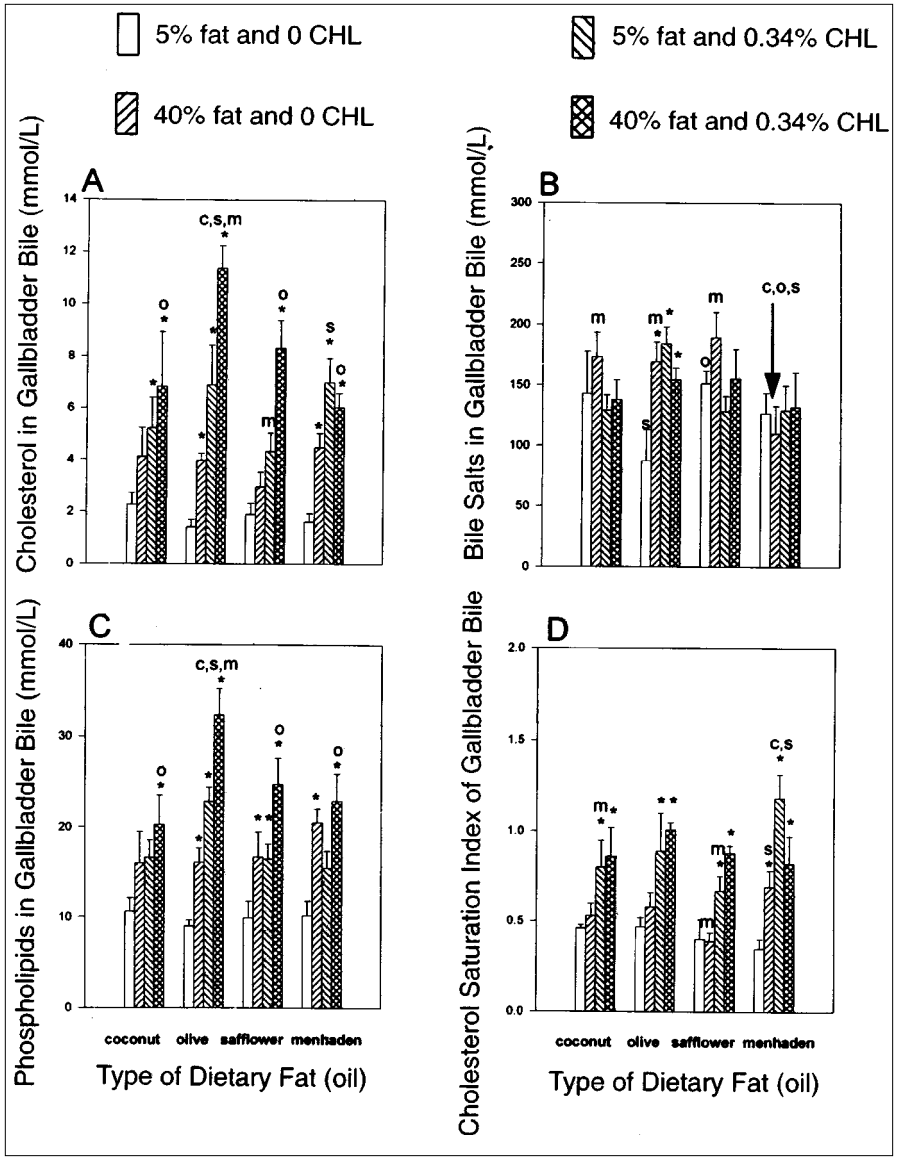

FIG. 3. Effect of dietary fat and cholesterol on the concentrations of lipids in gallbladder bile. A: Cholesterol. B: Bile salt. C: Phospholipid. D: Cholesterol saturation index. c, o, s, m, * and abbreviation as described in Figure 1.

PL concentrations in response to dietary CHL ($P = 0.0001$) or increased dietary fat ($P = 0.0001$), and they tended to mirror changes in biliary FC. The greatest increases in FC and PL concentrations in bile were seen in the animals fed diets containing CHL and high levels of olive oil. The concentrations of BS varied from diet to diet, but differences tended to be minimal, and there was no distinct effect which could be attributed to the type or amount of dietary fat or to the presence of CHL in the diet. As shown in Figure 3D, the highest values for CSI were obtained with diets containing CHL ($P = 0.0001$), although in general bile tended to remain unsaturated.

Analysis of the BS species in gallbladder bile by HPLC revealed that in the prairie dog, the predominant species are taurocholate (TCA) and taurochenodeoxycholate (TCDCA) (Fig. 4). A shift from TCA to TCDCA was associated with both supplementation with dietary CHL ($P = 0.0001$) and increases in dietary fat ($P = 0.0001$), with concurrent supplementation having an additive effect ($P = 0.005$). The ratio of TCA/TCDCA showed an inverse relationship to the concentration of FC in gallbladder bile ($r = -0.61$; $P = 0.0001$).

DISCUSSION

Many studies have examined the effects of dietary lipids on plasma lipoproteins, and other studies have demonstrated that dietary CHL and TG intake can also influence biliary lipid composition. The liver, of course, plays a critical role in determining how diet will ultimately affect lipid concentrations

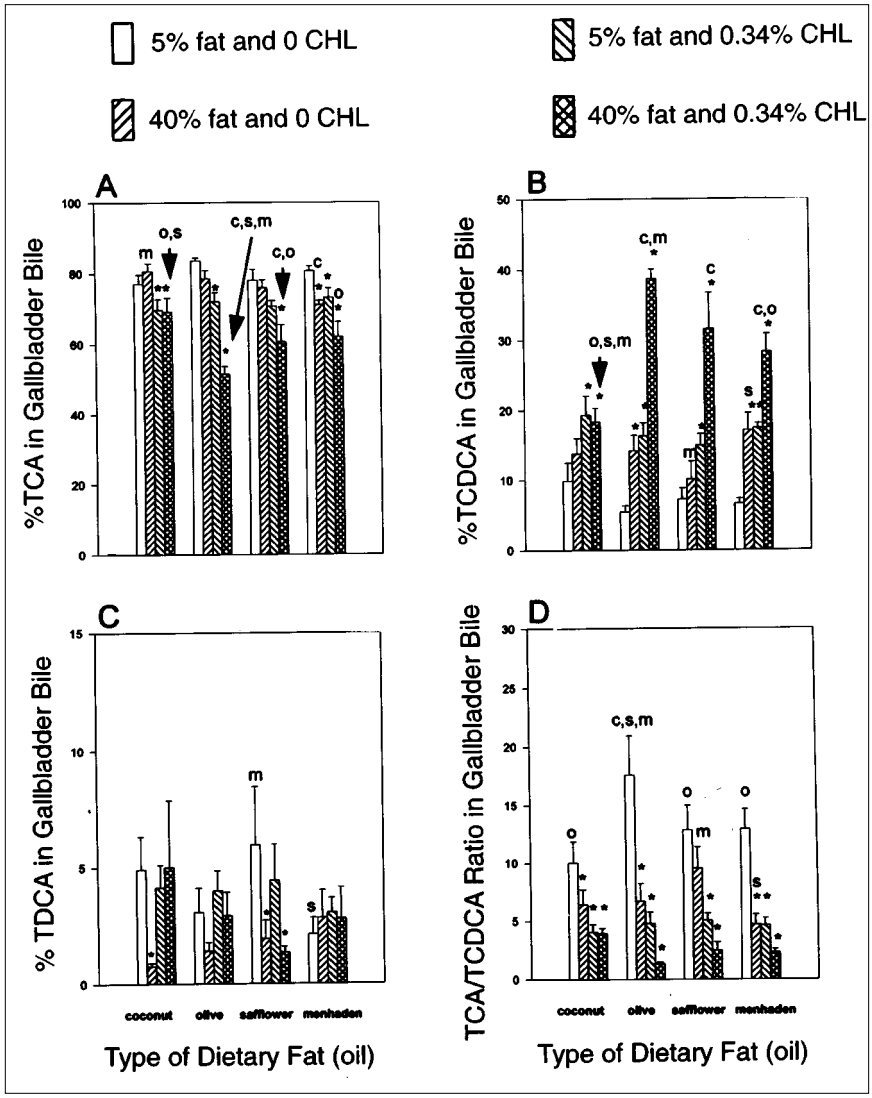

FIG. 4. Effect of dietary fat and cholesterol on the relative concentrations of bile salt species in gallbladder bile. A: Taurocholic acid (TCA). B: Taurochenodeoxycholic acid (TCDCA). C: Taurodeoxycholic acid (TDCA). D: Taurocholic acid/taurochenodeoxycholic acid (TCA/TCDCA) ratio. c, o, s, m, * and abbreviation as described in Figure 1.

in either plasma or bile. The studies reported here are somewhat unique because, first, they explored the impact of dietary CHL and TG on all three compartments (liver, plasma, and bile) simultaneously, and second, the effects of diet were examined by comparing 16 different diets in which the type of dietary TG, the amount of TG, and the amount of dietary CHL were systematically varied. In addition, the studies were designed to examine lipid concentrations prior to the formation of CHL gallstones, since the presence of stones in the gallbladder can potentially alter lipid metabolism. The major findings obtained from this experimental approach were as follows: (i) supplementation with dietary CHL resulted in increases in the CHL levels in liver, plasma, and bile, (ii) the effects of an increase in dietary fat were less extensive, with increases occurring primarily in biliary CHL, but also to some extent in hepatic CE, (iii) increases in both dietary CHL and fat frequently had a synergistic effect, resulting in the highest levels of plasma and biliary CHL, (iv) the type of dietary oil had a more profound effect on TG levels than on CHL, and (v) increases in hepatic FC concentrations were more limited than the increases in FC in bile or plasma.

Increased dietary fat consumption probably raised the input of CHL to the hepatic compartment. However, hepatic FC levels remained relatively normal, whereas the concentrations of biliary CHL and, to a lesser extent, hepatic CE increased. Larger increases in hepatic CHL levels, such as those in response to ingestion of CHL-supplemented diets, resulted in expansion of the hepatic FC pool. However, even in these cases, the increase in hepatic FC was modest compared to the dramatic elevations in biliary CHL and hepatic CE. Although neither the secretion of biliary CHL nor the synthesis of hepatic CE was measured directly, these results suggest that alterations in the levels of these two lipids are important for maintaining the hepatic FC pool. Furthermore, while there was evidence that input of total CHL to the hepatic compartment was increased by supplementation with either dietary fat or CHL, concurrent supplementation produced hepatic FC concentrations no higher than those resulting from CHL supplementation alone. However, the highest levels of hepatic CE and biliary CHL were observed in those animals receiving high levels of fat and CHL simultaneously. These findings imply that although it is possible to increase hepatic FC above normal levels if the input of CHL to the liver is high enough, there is a limit above which excess FC will either be excreted or converted to a storage form. Finally, the concentrations of plasma CHL became elevated only in the presence of severe increases in hepatic CHL, suggesting that the primary response to an increased input of CHL into the hepatic compartment is an augmented biliary CHL secretion.

The finding in this study that coconut oil raised plasma CHL concentrations in comparison to olive, safflower, and menhaden oils in the absence of dietary CHL supplementation is consistent with previous reports of the link between saturated fats and hypercholesterolemia (8–10), and also with studies demonstrating a hypocholesterolemic effect of monounsaturates (17–20) and n-6 (17,18) and n-3 polyunsaturates (22–24). On the other hand, in the presence of 0.34% dietary CHL, the concentrations of plasma CHL were elevated for all dietary oils. Given the prevalence of CHL in the human diet, these results support current recommendations for the reduction of total fat consumption, regardless of the degree of unsaturation. It should be noted that in this study the amount of CHL ingested per kg of body weight was probably about 10–12 times greater than in a "typical" American diet (41). The 0.34% CHL diet was chosen because it is a frequently used "lithogenic" diet in this animal model. However, in view of our findings, it would be of interest to examine the metabolic responses to a more moderate dietary CHL load.

Menhaden oil supplementation resulted in the lowest levels of plasma TG, similar to previous studies reporting a hypotriglyceridemic effect of marine n-3 polyunsaturated fats (21–23). Some controversy exists as to the mechanism by which this reduction in TG occurs. Several reports indicate that fish oil and n-3 polyunsaturated fatty acids reduce hepatic TG synthesis (42–44), while other evidence suggests that n-3 oils reduce plasma TG by inhibiting hepatic secretion (45,46). The present study suggests that fish oil decreases hepatic TG secretion, since the 40% menhaden oil diet resulted in low plasma TG concentrations in the presence of high levels of hepatic TG. However, as TG synthesis was not measured directly in this study, the possibility exists that both synthesis and secretion were reduced, with the change in synthesis masked by the accumulation of TG which would likely occur as the result of the inhibition of secretion.

Recent studies indicate that dietary CHL can induce hypertriglyceridemia in the rat *via* an increased synthesis of fatty acid and secretion of TG by the liver (47,48). However, in the present study, dietary CHL actually reduced the concentration of plasma TG for all oil groups. Furthermore, the only lipid parameter which failed to show a significant response to dietary CHL supplementation was the concentration of hepatic TG. A potential explanation is that in the prairie dog, rather than stimulating TG synthesis, CHL derived from the diet is converted in the liver to CE and preferentially secreted in very low density lipoprotein (VLDL) at the expense of TG.

Diet also influences the hepatic secretion of biliary lipids, particularly CHL. Increased biliary CHL secretion is probably the primary factor responsible for CHL cholelithiasis in western populations. In this study the concentration of biliary CHL increased as a result of dietary fat supplementation, although significant differences in comparison to the low fat, trace CHL animals were obtained for the olive and menhaden oil groups only. In light of previous studies which cited increases in biliary CHL in response to dietary saturated fats (12) as well as to n-6 (49,50) and n-3 polyunsaturated fats (51,52), it is interesting to note that in the present study, the highest concentrations of biliary CHL were obtained during CHL supplementation in the olive oil group. Since the predominant fatty acid contained in olive oil is oleic acid, these results suggest that an increased dietary intake of the n-9 monounsaturated fats may also result in an enhanced secre-

tion of CHL into bile. The saturation of bile with CHL was not substantially increased by supplementation with dietary fat alone, because there were corresponding increases in biliary PL. Biliary PL also increased in response to elevations in dietary CHL, but a proportionally greater increase in biliary CHL produced significant increases in CSI. Nevertheless, gallbladder bile generally remained unsaturated in this study due to the relatively brief duration of feeding of experimental diets. In our experience, supplementation with CHL at the level of 0.34% requires a minimum of 3–4 wk to induce CHL-saturated bile.

The concentrations of BS were not significantly influenced by diet in this study, although changes in BS secretory rate may have occurred. However, ingestion of dietary fat and CHL did induce alterations in the species of BS secreted, and these changes may have physiological significance as well. Chenodeoxycholic acid possesses a greater equilibrium CHL solubility than cholic acid (53), and relative increases in the concentration of the former in conjunction with increases in bile lithogenic index have been reported (54,55). In the present study, the TCA/TCDCA ratio significantly decreased with dietary CHL supplementation, in agreement with previous reports in humans (54), hamsters (56), and prairie dogs (29). Dietary fat supplementation also significantly decreased the TCA/TCDCA ratio for all oils tested. To our knowledge, this is the first evidence that dietary fat exerts an independent effect on the relative composition of BS species. Furthermore, there was a trend toward even lower ratios when dietary fat and CHL were increased concurrently, a situation which yielded the highest concentrations of biliary CHL. One possible explanation is that a shift toward TCDCA synthesis occurs whenever there is an increase in the hepatic FC pool and therefore a need to secrete more CHL into bile.

The changes in lipid concentration which were documented in this study could have occurred through several physiological mechanisms. Evidence exists that the regulatory pool of FC within the liver is maintained by coordination of the activities of hydroxymethylglutaryl-CoA reductase (57,58), acyl CoA-cholesterol acyltransferase (59,60), and cholesterol 7 α-hydroxylase (61,62). In addition, the hepatic LDL-receptor provides an important level of control in lipid homeostasis (63,64). Finally, the regulation of apoprotein synthesis influences the assembly and metabolism of the plasma lipoproteins (64,65). Modulation of any of these regulatory factors in response to diet is possible, and further work in each of these areas is currently underway in this laboratory. This study provided a broad examination of the overall effects of dietary fat and CHL on lipid concentrations, but more detailed information regarding the changes occurring at the molecular level will be crucial for a better overall understanding of the complexities of lipid homeostasis.

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The Influence of Dietary Lipids on the Composition and Membrane Fluidity of Rat Hepatocyte Plasma Membrane

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ABSTRACT: Weanling male Wistar rats were fed for five weeks on standard rat chow (23 g fat/kg diet) or one of four synthetic diets with butterfat, coconut oil, corn oil, or fish oil as the main lipid source (100 g fat/kg diet). In all diets, 10% of the fat was provided as corn oil to prevent essential fatty acid deficiency. Significant differences were observed in the saturated, monounsaturated, and polyunsaturated fatty acid composition, and in the ratio of cholesterol to phospholipid, in the hepatocyte membranes. The fluidity of hepatocyte plasma membranes was assessed using the fluorescence recovery after photobleaching technique and steady-state fluorescence anisotropy of diphenylhexatriene. No significant differences were found in the fluidity of plasma membranes between animals on the different fat diets, despite diet-induced changes in their fatty acid composition. However, the proportion of lipid free to diffuse in the plasma membrane varied with diet, being significantly greater ($P < 0.05$) in animals fed chow (63.7%), coconut oil (61.5%), and butterfat (57.6%) diets than in those fed the corn oil (47.3%) diet. Animals fed fish oil showed an intermediate (50.0%) proportion of lipid free to diffuse. The data support the hypothesis that dietary lipids can change both the chemical composition and lateral organization (lipid domain structure) of rat hepatocyte plasma membranes.
Lipids 32, 179–184 (1997).

The fatty acid composition of the cell membrane modulates its structure, fluidity, and function (1,2). Cellular functions, including production of eicosanoids and other secondary messengers (3–6), receptor function (7–9), and enzyme activity (10,11) may be altered by changes in the membrane composition. Such changes may be achieved through manipulation of dietary lipid intake (12,13). Membrane fluidity is influenced by changes in the level of unsaturation of the phospholipid fatty acyl chains (14,15), phosphatidylethanolamine ratios (16), cholesterol to phospholipid, or protein to lipid ratios (17–19) and fatty acyl chain length (20). The fluidity of the membrane has been shown to modulate cellular functions such as secretion, chemotaxis, signal transmission, and susceptibility to microorganism invasion (21–23).

In vivo, the ratio of saturated to unsaturated fatty acids and the amount of cholesterol in the membrane are closely regulated. This may represent a mechanism for maintaining membrane fluidity within certain limits for optimal membrane function known as "homeoviscous adaptation" (14,24). These mechanisms may be expected to influence the composition of the membrane following manipulation of dietary lipid intake, and hence resist attempts to alter membrane fluidity. Current evidence suggests that the cell membrane is not homogenous in character, but is composed of lipid "domains" which vary in size, composition, and, potentially, fluidity (11,25). Compositional changes may not therefore occur in a uniform manner throughout the membrane. Furthermore, changes in the fluidity of the domains may take place, without any change being measured in overall or "bulk" fluidity.

The present study uses steady-state anisotropy and fluorescence recovery after photobleaching (FRAP) to investigate the extent to which chronic changes in dietary lipid intake affect the composition and physical properties of rat hepatocyte plasma membranes. Steady-state anisotropy determines rotational motion and is sensitive to changes in the packing of the lipid molecules in the membrane, whereas FRAP measures the freedom of molecules to diffuse laterally in the plane of the membrane. Measurement of rotational diffusion provides information about molecular motion on a molecular length scale and in the case of FRAP, measurements of lateral diffusion provide information about molecular motion on a larger scale, determined by the dimensions of the illuminated spot on the membrane surface. Thus this latter measurement is sensitive to the presence of barriers to diffusion that may be present within the membrane, resulting in domain formation.

MATERIALS AND METHODS

Animals and diets. Twenty weanling male Wistar rats were maintained for five weeks on a diet composed of either standard rat chow (23 g fat/kg diet) (Special Diets Services,

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Abbreviations: C/PL, cholesterol to phospholipid; D, lateral diffusion coefficient; DPH, diphenylhexatriene; FRAP, fluorescence recovery after photobleaching; HBSS, Hank's balanced salts solution; ODAF, 5-*N*-(octadecanoyl) aminofluorescein; %R, percentage recovery.

Witham, Essex, United Kingdom), or one of four synthetic diets (100 g fat/kg diet) containing butterfat (Dairy Crest, Surbiton, Surrey, United Kingdom), coconut oil (Booker Foodstuffs, Dorset, United Kingdom), corn oil (Mazola/CPC, Esher, Surrey, United Kingdom), or fish oil (MaxEPA; Seven Seas Healthcare, Hull, United Kingdom) as the main lipid source. In all diets, 10% of the fat was provided as corn oil to prevent essential fatty acid deficiency. (For fatty acid composition of diets, see Table 1). The diets were otherwise identical and adequate in protein (177 g casein/kg diet, 3 g dL-methionine/kg diet), carbohydrate (285 g sucrose/kg diet, 285 g starch/kg diet), fiber (100 g cellulose powder/kg diet), vitamins and minerals (50 g vitamin E-free vitamin and mineral mixture/kg diet, (Special Diets Services), supplemented with appropriate levels of vitamin E to provide 90 mg vitamin E/kg of diet). Care was taken with respect to storage and allocation of the diets to minimize susceptibility to oxidation. The animals were individually housed in wire-bottomed cages at 22°C on a 12-h light/dark cycle, with free access to food and water.

Hepatocyte isolation. After five weeks, the livers were excised, macerated and hepatocytes isolated by incubation with Ca^{2+}/Mg^{2+}-free Hank's balanced salts solution (HBSS) (Sigma Chemical Company, Poole, Dorset, United Kingdom) for 5 min, followed by 10 mg/100 mL collagenase (Boehringer Ltd., Lewes, Sussex, United Kingdom) in Ca^{2+}/Mg^{2+}-containing HBSS (Sigma), at 37°C pH 7.4, for 20 min. Dispersed liver cells were filtered, washed, and resuspended in Ca^{2+}/Mg^{2+}-containing HBSS. This cell suspension was used for the isolation of plasma membranes and the measurement of lateral mobility of the membrane lipids.

Plasma membrane preparation. Rat liver plasma membranes were prepared according to the method of Loten and Redshaw-Loten (26).

Membrane composition. Total lipids were extracted from the isolated plasma membrane preparations by the method of Bligh and Dyer (27), and phospholipids separated from nonpolar lipids by thin-layer chromatography. Phospholipids were methylated by heating with boron trifluoride–methanol (Sigma), and the fatty acid derivatives separated on a gas chromatograph (Hewlett-Packard 5890 Series II, Palo Alto, CA). This method provided quantitative analysis of the fatty acids present by comparison with internal standards. Free cholesterol content was assayed enzymically using cholesterol oxidase (28,29).

Lateral mobility of membrane lipids. This was measured using the fluorescence recovery after photobleaching (FRAP) technique (30), using 5-*N*-(octadecanoyl) aminofluorescein (ODAF) (Molecular Probes, Eugene, OR) as the fluorescent probe. This technique measures the lateral diffusion coefficient (D) of a fluorescent lipid analogue that has been introduced into the plasma membrane. In addition, the proportion of membrane lipids that are mobile, i.e., free to diffuse out of the *ca*. 1 µm diameter measured region of the plasma membrane during the time course of the experiment, can be calculated from the data and expressed as a percentage recovery (%R) of fluorescence. A suspension of hepatocytes (50 µL) was incubated for 15 min on ice with 20 µL ODAF (33 µM), made up to 500 µL with Ca^{2+}/Mg^{2+}-containing HBSS. The labeled hepatocytes were centrifuged at 100 × *g* for one minute, washed twice with HBSS, and finally resuspended in 50 µL HBSS. A sample of this suspension was loaded by capillary action into a microslide (viewing path length 0.05 mm) (Camlab Ltd., Cambridge, United Kingdom) and was viewed on a Nikon Optiphot microscope equipped with a 100× objective lens and using brightfield or full-field epi-fluorescent illumination. Following selection and alignment of the target cells for FRAP measurement, a small region of the cell (1.3 μm^2) was illuminated using an attenuated laser beam (Coherent Innova 100, Palo Alto, CA). The FRAP measurement was computer-controlled and comprised a prebleach phase (*ca*. 0.5 s), a bleach period (7 ms) and a recovery phase (*ca*. 2 s). For each sample of hepatocytes, three sets of 10 FRAP measurements were taken, a different cell being used for each reading. Values for the diffusion coefficient and percentage recovery were calculated from each set of 10 FRAP measurements using methods described previously (30). The microslide was kept at a temperature of 37°C during the course of the experiment.

Membrane order. This was determined by fluorescence anisotropy of the probe diphenylhexatriene (DPH, Molecular Probes) under steady-state conditions using a spectrofluorometer (Perkin-Elmer LS-5, Norwalk, CT) coupled with an L-format polarization unit (31). DPH prepared as a 2-mM stock solution in tetrahydrofuran was stored refrigerated and protected from light. Equal volumes (0.5 mL) of hepatocyte plasma membranes (100 µg protein/mL) and DPH dispersion (2 µM) were mixed and incubated at 25°C for 30 min. Sample temperature was then adjusted and fluorescence measured over a range of temperatures with constant stirring. Temperature was monitored continuously using a digital thermometer with an accuracy of ±0.1°C. Excitation and emission wave-

TABLE 1
Fatty Acid Composition of Diets (g/kg of diet)

	Diet				
Fatty acid	Chow[a]	Butterfat[b]	Coconut oil[b]	Corn oil[b]	Fish oil
12:0	0.2	2.4	40.4	0.0	0.0
14:0	1.4	7.8	13.4	0.6	6.4
16:0	3.2	19.5	9.0	13.4	17.1
16:1n-7	1.0	1.9	0.4	0.3	8.9
18:0	0.4	8.0	2.3	2.2	4.0
18:1n-9	7.6	22.3	8.5	28.7	13.5
18:2n-6	7.1	5.8	6.3	47.8	8.6
183n-3	0.6	1.2	0.2	1.5	0.2
20:4n-6	1.3	0.0	0.0	0.0	1.4
20:5n-3	0.0	0.0	0.0	0.0	16.4
22:6n-3	0.0	0.0	0.0	0.0	11.5
U/S ratio[c]	3.3	0.8	0.2	4.8	2.2

[a]Special Diets Services (Witham, Essex, United Kingdom).
[b]From Reference 40.
[c]Unsaturated/saturated fatty acid ratio.

lengths were 355 and 428 nm, respectively. The results were corrected for light scattering (32,33).

The equation used to determine anisotropy (r) is given below:

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}} \quad [1]$$

where I_{VV} and I_{VH} are the emitted light components polarized vertically (V) and horizontally (H) with reference to the polarization orientation of the excitation light and G is an instrumental correction factor (30). Membrane fluidity is inversely proportional to anisotropy.

Statistics. All data were analyzed using unpaired Student's *t*-tests, with a probability level of 5% or less being accepted as statistically significant. Correlation coefficients were calculated by linear regression analysis.

RESULTS

Body weights and food intakes of the animals were recorded throughout the experiment, and no significant differences were found between the five dietary groups.

Plasma membrane phospholipid fatty acid compositions in rats fed the various diets are shown in Table 2. Considerable variation from the chow controls was observed. In chow-fed animals, the predominant fatty acid species were 16:0, 18:0, 18:2n-6, and 20:4n-6. In butter-fed animals, the membrane phospholipids had a significantly reduced 18:2n-6 content, with a concomitant increase in 18:0 and 18:1n-9. Coconut oil-fed rats demonstrated a significant depletion of 16:0 and 18:2n-6, and an increase in 18:0 and 18:1n-9 compared to chow controls, and had double the 20:4n-6 content of their membrane phospholipids. Feeding the corn oil diet elicited similar changes to the coconut oil diet, with a significant depletion of 16:0 and increased 18:0 and 20:4n-6 content. The effects of fish oil largely opposed the other experimental diets with significantly increased 16:0, 18:1n-9, 18:3n-3, 20:5n-3, and 22:6n-3 content being observed, relative to controls, with notable depletion of 18:0, 18:2n-6, and 20:4n-6 fatty acids.

The ratio of unsaturated to saturated fatty acids did not vary significantly from the control in any of the experimental groups. The ratio of n-3 to n-6 fatty acids in hepatocyte membrane phospholipids was significantly greater in the animals fed fish oil (0.75) than in any of the other experimental diets, or in chow-fed controls (0.13). Cholesterol to phospholipid (C/PL) ratios varied significantly from control in all rats fed experimental diets except those fed butterfat. Ratios were lower in coconut- and fish-oil fed animals, and higher in those fed corn oil. The C/PL ratio was negatively correlated with the ratio of n-3 to n-6 fatty acids in the membrane phospholipids ($r = 0.95$, $P < 0.05$).

Lateral diffusion coefficients in hepatocyte plasma membranes from the various dietary groups showed no significant variations from the chow-fed controls (Fig. 1). Lateral diffusion was positively correlated with the ratio of n-3 to n-6 fatty acids in the membrane phospholipids ($r = 0.89$, $P < 0.05$), and, to a lesser extent, negatively correlated with the C/PL ratio ($r = 0.76$, ns). However, the proportion of membrane lipids which were mobile as determined by FRAP analysis (%R) was influenced by dietary manipulation (Fig. 2). Corn oil-fed rats (47.3%) were found to have significantly reduced mobility relative to controls (63.7%).

Table 3 shows the values obtained for the fluorescence anisotropy (r) of DPH at 37°C in the hepatocyte plasma membranes of animals fed the different diets. Higher values indicate a smaller range and rate of motion of the DPH probe, suggesting a less fluid membrane. Values of r ranged from 0.1176 in the coconut oil-fed animals to 0.1455 in those ani-

TABLE 2
Fatty Acid Composition (% by weight of total fatty acids) of Hepatocyte Plasma Membrane Phospholipids[a]

	Diet				
Fatty acid	Chow	Butterfat	Coconut oil	Corn oil	Fish oil
12:0	0.1 ± 0.08	0.1 ± 0.08	0.1 ± 0.08	0.1 ± 0.06	0.1 ± 0.10
14:0	0.5 ± 0.14^{a}	0.3 ± 0.13^{a}	1.1 ± 0.08^{b}	0.6 ± 0.15^{a}	1.8 ± 0.14^{c}
16:0	35.5 ± 1.12^{c,d}	30.8 ± 2.01^{b,c}	23.2 ± 2.32^{a}	28.9 ± 0.58^{a,b}	41.6 ± 1.96^{d}
16:1n-7	0.8 ± 0.11^{a}	0.5 ± 0.12^{a}	0.7 ± 0.12^{a}	0.4 ± 0.15^{a}	1.9 ± 0.12^{b}
18:0	17.9 ± 0.52^{b}	26.3 ± 2.09^{c}	26.1 ± 1.26^{c}	23.3 ± 1.26^{c}	11.3 ± 1.09^{a}
18:1n-9	4.0 ± 0.13^{a}	6.8 ± 0.58^{c}	5.4 ± 0.46^{b}	5.2 ± 0.23^{a,b}	14.2 ± 0.18^{d}
18:2n-6	17.8 ± 0.24^{c}	12.5 ± 0.84^{b}	10.6 ± 1.11^{a,b}	15.8 ± 1.04^{c}	9.2 ± 0.86^{a}
18:3n-3	0.2 ± 0.15^{a}	0.2 ± 0.11^{a}	n.d.	n.d.	0.6 ± 0.13^{b}
20:4n-6	12.1 ± 0.89^{b}	15.1 ± 0.24^{c}	23.4 ± 0.92	19.2 ± 0.82^{d}	3.5 ± 0.33^{a}
20:5n-3	0.7 ± 0.13^{b}	0.1 ± 0.03^{a}	n.d.	n.d.	4.5 ± 0.30^{c}
22:6n-3	2.9 ± 0.30^{b}	1.6 ± 0.07^{a}	4.1 ± 0.05^{c}	1.6 ± 0.11^{a}	5.1 ± 0.34^{d}
U/S ratio[b]	0.7 ± 0.03	0.6 ± 0.05	0.9 ± 0.09	0.8 ± 0.05	0.7 ± 0.04
C/PL ratio[c]	0.4 ± 0.01^{b}	0.4 ± 0.03^{b}	0.3 ± 0.02^{c}	0.5 ± 0.03^{a}	0.2 ± 0.01^{d}

[a]Values are expressed as mean ± SEM for four observations per dietary group. Values in the same row with different superscripts are significantly different ($P < 0.05$); n.d., not detected.
[b]Unsaturated/saturated fatty acid ratio.
[c]Cholesterol/phospholipid ratio.

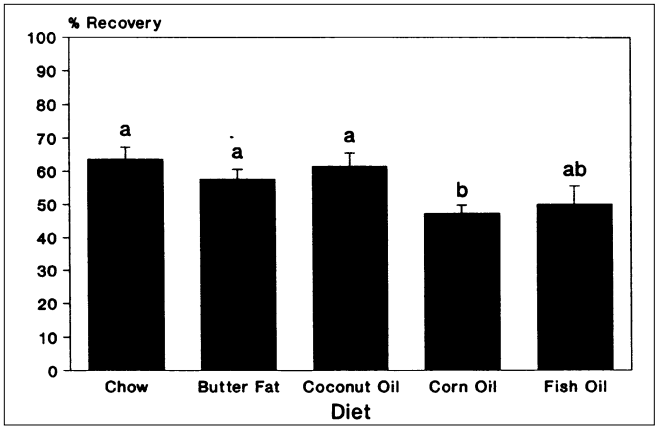

FIG. 1. Lateral diffusion rates of hepatocyte plasma membrane phospholipids. Lateral diffusion rates of hepatocyte plasma membrane phospholipids were measured, as an indication of membrane fluidity, using fluorescence recovery after photobleaching. The figures represent the mean + SEM for four observations per dietary group. No significant differences were found between the dietary groups.

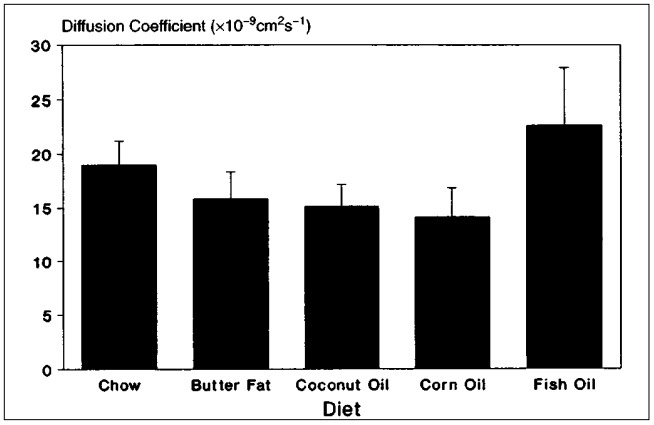

FIG. 2. Percentage recovery of hepatocyte plasma membrane phospholipids. Percentage recoveries of hepatocyte plasma membrane phospholipids were measured, as an indication of membrane lipid lateral mobility, using fluorescence recovery after photobleaching. The figures represent the mean + SEM for four observations per dietary group. Figures with unlike letters are significantly different ($P < 0.05$).

mals fed corn oil diets. There were no significant differences in membrane fluidity between the dietary groups.

DISCUSSION

Plasma membrane fatty acid composition is determined by the availability and biotransformation of stearic acid (18:0) to oleic acid (18:1n-9, and oleic, linoleic (18:2n-6) and linolenic (18:3n-3) acids to polyunsaturated fatty acids (1). In the present study, quantities of oleic acid were increased, and those of linoleic acid decreased, in animals fed the experimental diets compared with chow-fed controls. Quantities of arachidonic acid (20:4n-6) were significantly different in all diets, increasing in content in the order fish oil < chow < butterfat < corn oil < coconut oil. Membrane phospholipids from fish oil-fed animals contained significantly greater quantities of n-3 fatty acids, and less n-6 fatty acids, than those from any of the other diets. Thus the fatty acid composition of the diet significantly influenced that of the hepatocyte membrane phospholipids.

The lateral diffusion coefficient of ODAF in the hepatocyte plasma membrane was not influenced significantly by the diet-induced changes in the fatty acid composition of hepatocyte membrane phospholipids. Lateral diffusion coefficients were positively correlated with the ratio n-3 to n-6 fatty acids, and, to a lesser extent, negatively correlated with the ratio of C/PL in the membranes. The observed effects of the experimental diets on C/PL ratios were consistent with results from other studies (7,24,34). Since cholesterol increases the order of lipids in the membrane, leading to reduced fluidity (13), the negative correlation found between diffusion rates and C/PL ratios was expected, and agrees with results from other studies (17,18).

Steady-state fluorescence anisotropy of DPH in the hepatocyte plasma membranes was also not influenced significantly by diet. This result is consistent with other findings (35,36), and suggests that packing of the lipid molecules of the plasma membrane is largely unaffected by the diets. It is also interesting to note that both FRAP and anisotropy measurements provide consistent results. For example, hepatocyte cell membranes from animals fed corn oil diets exhibit both the lowest rates of lipid lateral diffusion and the highest values for fluorescence anisotropy, suggesting that these membranes are the least fluid. This result may be due to the C/PL ratio of the membranes, which is highest in the corn oil group.

Membrane fluidity is not influenced by dietary manipulation, of the type described in the present study probably due to homeoviscous adaptation. There is evidence to suggest that homeoviscous adaptation occurs *in vivo* (14,24), maintaining membrane fluidity within narrow limits. To a certain extent, this will be achieved through the specificity of the *sn*-1 and

TABLE 3
Fluorescence Anisotropy (*r*) of Hepatocyte Plasma Membranes at 37°C[a]

Diet	Chow	Butterfat	Coconut oil	Corn oil	Fish oil
r	0.1276 ± 0.0069	0.1201 ± 0.0038	0.1176 ± 0.0097	0.1455 ± 0.0055	0.1219 ± 0.0080

[a]Values are expressed as mean ± SEM for four observations per dietary group. No significant differences were found between the dietary groups.

sn-2 positions of the major phospholipids for saturated and unsaturated fatty acids, respectively (13). This is reflected in the lack of any significant differences between the unsaturated to saturated fatty acid ratios in the membrane phospholipids in animals fed the different fat diets, and is consistent with results from other studies (13,24,37). Homeoviscous adaptation may be involved in the "fine tuning" of membrane fluidity, by regulating membrane cholesterol content, phospholipid class distribution, and/or the activities of the membrane-bound desaturase and elongase enzymes. These activities may be regulated by membrane fluidity itself *via* a negative feedback mechanism (20,38). The existence of homeoviscous adaptation implies that maintenance of membrane fluidity, within certain limits, is important for membrane function.

Homeoviscous adaptation may also be an important factor to consider when examining plasma membrane composition. The composition of the membrane will be determined both by the diet and by the changes initiated by the cell in response to any change in membrane fluidity. Whether a homeoviscous adaptation response has occurred in the present study, and to what extent the membrane compositional changes are due to diet alone, or to a combination of diet and homeoviscous adaptation, is unclear. Furthermore, owing to the domain-like nature of the plasma membrane, it is possible that the fluidities of the various membrane domains may change without a significant change in the overall fluidity. The fluidity of certain areas may therefore be significantly influenced by manipulation of dietary lipids, although no overall change is detected.

Although there were no significant differences observed in plasma membrane diffusion coefficients between the dietary groups, differences were seen in the %R of the membranes. Hepatocyte membranes from animals fed corn oil had a significantly lower value for %R than those fed chow, coconut oil, or butterfat. This would seem to indicate that there was a greater proportion of immobile lipid in hepatocyte cell membranes from animals fed the corn oil diet. This change in the physical state of the membrane may have functional significance. The low value for %R found in hepatocyte plasma membranes from corn oil-fed animals may have been expected, since these membranes have the greatest C/PL ratio and cholesterol has been shown to rigidify membranes (17,18). The extra cholesterol is probably not, however, evenly distributed throughout the membrane, leading to "cholesterol-poor" (relatively mobile) domains (25). As the cholesterol content of the membrane increases, so does the size of the cholesterol-rich domains, leading to a greater proportion of immobile lipid, and consequently a lower %R. Nevertheless, the %R of hepatocyte membranes from animals fed the fish oil diet was not significantly greater than that found in the corn oil-fed animals, despite the former having the lowest C/PL ratio of any of the dietary groups. Evidence suggests, however, that the highly unsaturated phospholipids associated with membranes from animals fed fish oil are unlikely to associate with cholesterol, and may thus create extremely fluid domains containing mostly phospholipid fatty acyl group which are highly unsaturated and more rigid cholesterol-rich domains leading to no overall change in membrane fluidity (39). Although there is less cholesterol overall in fish oil-supplemented membranes, it is confined to specific immobile domains, and hence a lower %R is determined by FRAP. This phenomenon might be thought to result in greater interdomain variation in hepatocyte plasma membranes from animals on the fish oil diet when compared to animals on the other diets. The results of the present study suggest that this is indeed the case.

In summary, rat hepatocyte plasma membrane composition can be altered by feeding animals diets of different fatty acid composition. Diet-induced membrane compositional changes do seem to influence, to a limited extent, the lateral diffusion of membrane lipid but significantly alter the lipid domain structure of the cell membrane. Whether these changes in the chemical composition and physical state of the membrane have any significant functional implications remains to be determined.

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Early Dietary Intervention with Structured Triacylglycerols Containing Docosahexaenoic Acid. Effect on Brain, Liver, and Adipose Tissue Lipids

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ABSTRACT: Newborn rats were fed liquid diets containing 7 wt% fat in which 3.8% of the total fatty acids were 22:6n-3. The fats were either a specific structured oil with 22:6n-3 mostly located in the *sn*-2 position or a randomized oil with 22:6n-3 equally distributed in the triacylglycerol (TAG) molecules. The oils were manufactured by interesterification of fish oil TAG with free fatty acids from butterfat. The pups were tube-fed three times a day and stayed with their dams during the night. After 14 d they were fed solid diets containing the same oils for the next 7 d. A reference group stayed with the dams and received ordinary rat chow at weaning. In general no significant differences between the two dietary treatments were observed in the tissues examined except for adipose tissue. The levels of 22:6n-3 were significantly increased in brain phosphatidylcholines (PC) and phosphatidylserines (PS) of both experimental groups compared with the reference group after three weeks, whereas no differences were found in brain phosphatidylethanolamines (PE) and phosphatidylinositols (PI). In all groups and all phospholipids examined, the levels of 20:4n-6 generally decreased from 1 to 3 wk and were significantly lower in the experimental groups compared with the reference group at 3 wk except for PI. In liver, PC and PE 22:6n-3 remained constant in the experimental groups but decreased significantly in the reference group, whereas in liver PS 22:6n-3 increased in all groups, but reached significantly higher levels in the experimental groups than in the reference group. In adipose tissue, 22:6n-3 increased in the experimental groups during the study period, but decreased in the reference group, suggesting that a surplus of dietary 22:6n-3 was stored.
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Polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (22:6n-3), are present in high amounts in the central nervous system (CNS) (1). They accumulate during the first 15 d after birth in the rat (2) and in humans in the fetal state and the first 6–10 mon after birth (3). The newborn rat is therefore a relevant model for the pre-term infant.

The biochemical significance of the enrichment in 22:6n-3 in the CNS is unclear. It takes, however, up to three generations to introduce n-3 deficiency in rats (4), indicating a strong ability to maintain these fatty acids in the brain and CNS. Prenatal deficiency in n-3 PUFA affects the fatty acid composition of the brain by reducing, in particular, the levels of 22:6n-3 (5,6), which correlates with reduced visual ability in rhesus monkeys (7). Brain fatty acid profiles of growing animals following recovery from n-3 fatty acid deficiency change very slowly compared to other tissues in both rats and monkeys (4,7). The adult brain is resistant to changes in dietary fatty acid composition (8), whereas the developing brain is much more plastic (9).

It is not known whether an optimal level of 22:6n-3 in brain phospholipids exists or which phospholipids are most important. In this context we have demonstrated that feeding fish oil as a source of n-3 PUFA affects rat brain phospholipids more than dietary flaxseed oil (10). We have also recently shown that the lymphatic absorption of a PUFA is enhanced when the PUFA is situated in the *sn*-2 position in the dietary TAG both under normal conditions (11) and in pancreatic insufficiency (12), and that the TAG structure of dietary fats influences the clearance rate of chylomicrons as well as the tissue uptake (13).

Interesterified oils made from medium-chain TAG and long-chain TAG have proven beneficial in patients with lipid malabsorption symptoms providing both essential fatty acids and readily oxidizable energy in the form of medium-chain fatty acids (MCFA) (14). Since the lipases in the gastrointestinal tract are *sn*-1/3 specific and enhanced absorption of the fatty acid in the *sn*-2 position is observed (11), specific structured TAG made by enzymatic interesterification, which preserves the PUFA in the *sn*-2 position, may improve the availability of PUFA. In the present investigation we therefore compared the dietary effect of a specific structured oil with 22:6n-3 located in the *sn*-2 position and a randomized oil, with the same fatty acid profile but with 22:6n-3 distributed evenly in the TAG molecule, on the accumulation of (n-3)

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Abbreviations: CNS, central nervous system; MCFA, medium-chain fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SCFA, short-chain fatty acids; TAG, triacylglycerol; TLC, thin-layer chromatography.

PUFA in the brain phospholipids of the newborn rat. The specific structured oil contained short- and medium-chain fatty acids from butter in the 1- and 3-positions. To examine the general accretion of 22:6n-3 in other organs or tissues, liver phospholipids and adipose tissue TAG were also examined.

MATERIALS AND METHODS

Synthesis of the specific structured and the randomized oil. The specific structured oil (Table 1) was manufactured by interesterification of a fish oil (Thyborøn Fiskeolieindustri A.m.b.a., Thyborøn, Denmark) and free fatty acids from butterfat using an *sn*-1/3 specific lipase (Lipozyme®; Novo Nordisk A/S, Bagsværd, Denmark) (15,16). By this procedure, long-chain PUFA were maintained in the *sn*-2 position whereas short- and medium-chain fatty acids from the butterfat were incorporated into the *sn*-1/3. The randomized oil (Table 1) was made from the specific structured oil by interesterification with sodium methoxide (17). The fatty acid compositions of the *sn*-2 position (Table 1) were determined following regiospecific Grignard degradation (11).

TABLE 1
Fatty Acid Composition of Dietary Oils

Fatty acids	Structured oil		Randomized oil		Milk
	TAG[a]	2-Pos[b]	TAG	2-Pos	TAG
4:0	2.52	n.d.	2.48	n.d.	n.d.
6:0	2.19	n.d.	2.23	n.d.	0.23
8:0	1.21	0.63	1.26	0.95	7.57
10:0	2.30	0.97	2.37	1.02	19.05
12:0	2.72	1.98	2.77	1.89	13.34
S/M FA[c]	10.94	3.61	11.11	3.86	40.19
14:0	10.04	9.98	9.99	9.39	9.74
14:1n-7	0.99	0.90	0.91	0.73	n.d.
15:0	0.40	0.79	0.36	0.53	n.d.
16:0	23.71	23.86	23.42	25.65	16.95
16:1n-7	4.09	4.14	4.33	3.79	1.25
17:0	0.52	0.39	0.64	0.46	n.d.
18:0	6.57	6.75	6.63	7.80	1.96
18:1	21.79	22.70	21.10	26.13	10.83
18:2n-6	3.39	4.19	3.43	3.62	13.50
18:3n-3	0.84	1.97	1.29	1.47	0.82
20:0	2.37	2.54	2.42	1.30	0.06
20:1n-9	2.90	3.11	2.85	2.89	0.20
20:2	0.07	0.09	0.06	0.06	n.d.
20:3n-6	0.09	n.d.	0.07	n.d.	0.48
20:4n-6	0.11	0.16	0.12	0.09	1.77
22:0	0.27	n.d.	0.25	n.d.	n.d.
20:5n-3	3.43	4.83	3.35	3.59	n.d.
22:1	3.40	3.40	3.32	4.81	0.35
22:5n-3	0.38	0.45	0.36	0.29	0.29
22:6n-3	3.70	6.18	3.98	3.57	0.66

[a]Fatty acid composition of triacylglycerols (TAG). Values are in mole%, averages of two determinations.
[b]Fatty acid composition in the *sn*-2 position, determined by Grignard degradation.
[c]Short- and medium-chain fatty acids (S/M FA): 4:0 to 12:0; n.d., not detected.

Animals. Pregnant Wistar rats (Møllegård Breeding Centre A/S, Ll; Skensved, Denmark) were housed in plastic cages and provided with tap water *ad libitum*. Room temperature was maintained at 24°C, the relative humidity was 45%, and the light/dark cycle was 12/12 h. Following delivery, dams and pups were divided into three groups. Two groups each of 4 dams and 24 pups received diets with structured oil or randomized oil, respectively, and a reference group of 3 dams each with 8 pups received rat chow (Altromin C0199; Altromin Inc., Lange, Germany). The chow contained 4 wt% fat with the following fatty acid profile: 14:0, 1.1%; 16:0, 27.5%; 18:0, 1.4%; 18:1, 14.4%; 18:2, 50.2%; 18:3, 4.9%). The pups stayed with their dams the first two days. At 8 a.m. on the third day, pups and dams were separated, and the pups were placed under a lamp at 30°C. At 10 a.m., and at 1 p.m. and 4 p.m., the pups were weighed and then fed 0.1 g liquid diet (see below) through a polyethylene tube mounted on a glass pipette. After the last feeding at 4 p.m., the pups were returned to their dams where they stayed until next morning. The weight of the feed per meal was gradually increased during the experimental period to 0.3 g. This feeding regimen continued for two weeks, whereafter they received a solid diet (see below) containing the same oils as the liquid diets. The pups in the reference group stayed with their dams throughout the feeding period, and were given rat chow at the time of weaning. On the third day (day 1 in the feeding regimen) before the feeding started, and after 1 and 3 wk five pups were decapitated and brain, liver, and adipose tissue dorsal to the kidneys were immediately excised and frozen in liquid nitrogen.

Liquid diets. Powder diet for rats (Altromin C189a without fat; Altromin Inc., Lange, Germany) was mixed with water and the manufactured oil in the ratio 60 g powder/15 g oil/150 mL water and mixed overnight with a magnetic stirrer. The composition of diet was (in wt%) protein, 8.4%; carbohydrates, 14.3%; fat, 6.7%.

Rat milk. The dams of the reference group were milked on day 5 and the fatty acid composition was determined (18) (Table 1).

Solid diets. After weaning, the pups were fed *ad libitum* a diet composed of (in wt%): 40% starch, 20% fat (the manufactured oils), 20% casein, 10% sucrose, 5% salt mixture, 4% cellulose powder, 0.5% choline chloride, and 0.5% vitamin mixture (19).

Analyses of tissue lipids. Brain, liver, and adipose tissue were extracted according to Folch *et al*. (20). Phospholipid classes in brain and liver were separated by thin-layer chromatography (TLC) (10) and methylated with BF_3 (21). Adipose tissue TAG was isolated by TLC (22), and methylated with KOH (23). The fatty acid methyl esters were analyzed by gas–liquid chromatography using a Fisons Instruments HRGC Mega 2 series 8560 gas chromatograph with flame-ionization detection (Fisons Instruments, Milano, Italy), and a BPX-70 fused silica capillary column (25 m × 0.22 mm i.d.) (SGE, Melbourne, Australia). On-column injection was applied. Initial oven temperature was 40°C for 2 min, followed by a tempera-

ture gradient of 10°C/min until 150°C, followed by a gradient of 3°C/min until 215°C, followed by a gradient of 5°C/min until a final temperature of 220°C which was maintained for 5 min. Identification was made by comparison with authentic standards (Nu-Chek-Prep Inc., Elysian, MN). Only fatty acids contributing more than 0.5% of total fatty acids are shown in the tables, except for the potential 22:6n-3 precursor 20:5n-3.

Statistics. Fatty acid determinations were compared by one-way analysis of variance, followed by Newman-Keuls Multiple Range Test. Testing was made on a 5% significance level.

RESULTS

Animals. The differences between the diets affected neither body weight nor the weights of organs examined.

Brain phospholipids. After 1 wk no differences in n-3 PUFA of brain phospholipids were found, whereas at 3 wk both experimental diets increased the levels of 22:6n-3 in phosphatidylserine (PS) (Table 2) and phosphatidylcholine (PC) (Table 3) significantly compared with the reference group and 0 wk, but there were no differences between the two experimental groups. No effects of the diets were found in phosphatidylethanolamine (PE) (Table 2) and phosphatidylinositol (PI) (Table 3), but all groups doubled the 22:6n-3 level in PI. At three weeks, there were small but significant incorporations of 20:5n-3 in the experimental groups. In general larger changes in n-3 PUFA with time were observed in the experimental groups than in the reference group. In all groups and all phospholipids, 20:4n-6 decreased with time. The changes were similar in the two experimental groups and the final levels were significantly lower than in the reference group. In the experimental groups, the levels of 22:4n-6 decreased significantly in PE, whereas they remained constant in the reference group. Also 22:5n-6 was significantly reduced in PE. The level of 18:1 generally increased during the experiment in all groups and all phospholipids. Only in PS was a difference between the experimental groups and the reference group observed. The levels of 16:0 decreased in all groups with time, whereas 18:0 increased (PC) or was unaffected (PE, PS, and PI) by time and diets.

Liver phospholipids. In general only minor effects related to TAG structure were found. After 1 wk the levels of 22:6n-3 had increased significantly in PS, whereas they remained constant in PE and PC during the experiment (Table 4). The reference group also increased the 22:6n-3 in PS, but to a significantly smaller extent and decreased the levels in PC and PE. No effects of TAG structure were seen. Major replacements of 20:4n-6 by 20:5n-3 were observed in PC and PE and were most pronounced in the group fed the randomized TAG. Decreases in 20:4n-6 in PS for all groups were followed mostly by incorporation of 22:6n-3 and only moderate levels of 20:5n-3 as a function of time. Linoleic acid increased in the reference group with time, but not in the experimental groups. A general increase of 18:1n-9 in all groups with time was observed for PC and PE. Saturated fatty acids, i.e., 16:0 and 18:0, were little affected by the diets and the time. In the reference group a significant decrease in 16:0 in PC was found.

Adipose tissue. The TAG structure had some effect on the deposition of n-3 PUFA. The highest levels of 22:6n-3 and 20:5n-3 were found in the group fed randomized fat. The dietary fats also increased 16:1, 18:1 and 20:1, and decreased 18:2n-6 and 20:4n-6. At the start the adipose tissue contained 14% 10:0 plus 12:0, which significantly decreased during the experiment. In the reference group 18:2n-6 accumulated.

DISCUSSION

In the present experiment, we examined if it was possible to increase the accumulation of 22:6n-3, especially in the brain of newborn rats, by dietary intervention using two different

TABLE 2
Fatty Acid Composition of Rat Brain PE and of Rat Brain PS

	PE							PS						
	Start	Reference group		Structured oil		Randomized oil		Start	Reference group		Structured oil		Randomized oil	
Diet	0 wk	1 wk	3 wk	1 wk	3 wk	1 wk	3 wk	0 wk	1 wk	3 wk	1 wk	3 wk	1 wk	3 wk
16:0[a]	11.76^{b,c,d}	12.42^{c,d}	9.30^{a}	12.78^{d}	10.67^{a,b,c}	11.48^{b,c,d}	10.26^{a,b}	2.78^{b,c}	2.47^{a,b}	3.20^{b,c}	3.68^{c}	1.65^{a}	2.57^{a,b}	1.59^{a}
16:1	0.41	0.30	0.44	0.28	0.21	0.29	0.25	0.34^{c}	0.25^{b,c}	0.11^{a,b}	0.24^{b,c}	0.07^{a,b}	0.21^{a,b,c}	0.04
18:0	23.72	23.86	24.51	24.59	24.36	24.25	24.42	41.09^{b}	40.98^{b}	38.47^{a}	40.02^{a,b}	40.45^{b}	42.17^{b}	40.89^{b}
18:1	7.86^{a}	8.21^{a}	15.14^{b}	8.67^{a}	16.11^{b}	8.69^{a}	15.53^{b}	4.89^{a}	5.86^{a}	14.77^{c}	5.75^{a}	12.32^{b}	6.26^{a}	12.56^{b}
18:2n-6	0.43^{b,c}	0.51^{c}	0.31^{a,b}	0.47^{c}	0.29^{a,b}	0.57^{c}	0.25^{a}	0.22^{b,c}	0.22^{b,c}	0.21^{b,c}	0.28^{c}	0.15^{a,b}	0.09^{a}	0.12^{a,b}
20:1	0.08^{a}	0.12^{a}	1.26^{b}	0.14^{a}	1.46^{b,c}	0.17^{a}	1.66^{c}	0.10^{a}	0.10^{a}	0.53^{b}	0.06^{a}	0.70^{c}	0.05^{a}	0.72^{c}
20:3n-6	0.40^{a}	0.51^{a,b}	0.59^{b}	0.48^{a,b}	0.66^{b}	0.56^{b}	0.58^{b}	0.47^{a}	0.64^{b,c}	0.62^{b,c}	0.59^{b,c}	0.66^{c}	0.53^{a,b}	0.60^{b,c}
20:4n-6	20.22^{c}	20.60^{c}	15.55^{b}	19.81^{c}	13.07^{a}	20.49^{c}	13.34^{a}	7.83^{c}	7.75^{c}	4.96^{b}	6.89^{c}	3.01^{a}	7.27^{c}	3.08^{a}
20:5n-3	0.07^{a}	0.05^{a}	0.07^{a}	0.04^{a}	0.26^{b}	0.04^{a}	0.24^{b}	0.04^{a}	0.03^{a}	0.08^{a}	—^{a}	0.25^{b}	—^{a}	0.18^{b}
22:4n-6	6.01^{a}	5.78^{a}	6.08^{a}	5.80^{a}	4.81^{b}	6.19^{a}	4.97^{b}	7.65^{c}	6.73^{b}	4.59^{a}	7.00^{b}	4.17^{a}	6.50^{b}	4.26^{a}
22:5n-6	2.92^{a}	2.20^{b}	1.31^{c}	2.22^{b}	0.83^{d}	2.37^{b}	0.88^{d}	4.08^{c}	3.09^{b}	2.19^{a}	3.32^{b}	2.11^{a}	3.28^{b}	2.03^{a}
22:5n-3	0.50^{b}	0.53^{b,c}	0.37^{a}	0.54^{b,c}	0.75^{d}	0.60^{c}	0.81^{d}	0.70^{c}	0.70^{c}	0.31^{a}	0.65^{c}	0.71^{c}	0.53^{b}	0.69^{c}
22:6n-3	24.48	23.72	23.85	23.11	25.20	23.20	25.75	28.41^{a}	29.99^{a}	28.74^{a}	30.22^{a}	32.10^{b}	30.02^{a}	31.92^{b}

[a]For each phospholipid and each fatty acid: Values (mean) not bearing the same superscript letter are significantly different at $P < 0.05$. If no superscript appears, values are not different; $n = 5$ in each data set; PE, phosphatidylethanolamine; PS, phosphatidylserine.

TABLE 3
Fatty Acid Composition of Rat Brain PC and of Rat Brain PI

	PC							PS						
	Start	Reference group		Structured oil		Randomized oil		Start	Reference group		Structured oil		Randomized oil	
Diet	0 wk	1 wk	3 wk	1 wk	3 wk	1 wk	3 wk	0 wk	1 wk	3 wk	1 wk	3 wk	1 wk	3 wk
14:0[a]	3.93^{d}	3.42^{c}	0.60^{a}	2.95^{b}	0.61^{a}	2.81^{b}	0.57^{a}	0.52^{b,c}	0.32^{a,b,c}	0.12^{a}	0.63^{c}	0.29^{a,b,c}	0.28^{a,b,c}	0.17^{a,b}
16:0	56.95^{b}	57.24^{b}	47.73^{a}	57.03^{b}	46.29^{a}	56.78^{b}	47.00^{a}	7.75	7.60	7.35	7.87	8.10	8.88	7.18
16:1	2.51^{c}	1.91^{b}	0.31^{a}	1.70^{b}	0.31^{a}	1.63^{b}	0.31^{a}	0.35	0.34	0.04	0.61	0.18	0.28	0.08
18:0	5.88^{a}	6.31^{a,b}	11.79^{c}	6.67^{b}	12.72^{d}	6.82^{b}	12.26^{c,d}	37.92	37.38	38.34	39.21	37.86	38.22	38.18
18:1	19.11^{a}	19.03^{a}	25.88^{b}	19.39^{a}	26.18^{b}	19.68^{a}	25.85^{b}	7.73^{a}	8.19^{a}	13.77^{b}	7.35^{a}	13.45^{b}	8.54^{a}	12.37^{b}
18:2n-6	1.09^{c}	1.29^{c}	0.88^{b}	1.20^{c}	0.61^{a}	1.29^{c}	0.70^{a,b}	0.33	0.55	0.13	0.50	0.17	0.23	0.14
20:1	0.22^{a}	0.26^{a}	0.77^{b}	0.29^{a}	1.04^{b}	0.32^{a}	1.04^{b}	0.09	0.11	0.13	0.17	0.27	—	0.20
20:3n-6	0.32^{a,b,c}	0.38^{d,e}	0.28^{a}	0.37^{c,d,e}	0.33^{b,c,d}	0.40^{e}	0.30^{a,b}	0.34^{a,b}	0.45^{b}	0.37^{a,b}	0.18^{a}	0.42^{a,b}	0.19^{a}	0.41^{a,b}
20:4n-6	6.00^{b}	6.21^{b,c}	6.46^{c}	6.39^{c}	5.41^{a}	6.21^{b,c}	5.62^{a}	37.42^{b}	36.71^{b}	29.18^{a}	35.62^{b}	29.26^{a}	36.86^{b}	31.88^{a}
20:5n-3	0.01^{a}	—^{a}	0.02^{a}	0.01^{a}	0.10^{b}	0.03^{a}	0.09^{a,b}	0.13^{b}	0.13^{b}	—^{a}	0.06^{a,b}	0.39^{c}	—^{a}	0.36^{c}
22:4n-6	0.56^{a}	0.53^{a}	0.67^{b}	0.59^{a}	0.53^{a}	0.58^{a}	0.55^{a}	0.91	0.95	1.06	0.89	0.65	0.87	0.61
22:5n-6	0.23^{a,b}	0.18^{a}	0.29^{c,d}	0.22^{a}	0.33^{d}	0.23^{a,b}	0.28^{b,c}	0.41	0.37	0.42	0.36	0.23	0.14	0.18
22:5n-3	0.06^{a}	0.06^{a}	0.05^{a}	0.08^{a}	0.15^{b}	0.07^{a}	0.13^{b}	0.05^{a,b}	0.10^{a,b}	0.06^{a,b}	0.05^{a,b}	0.13^{b}	—^{a}	0.10^{a,b}
22:6n-3	2.36^{a}	2.41^{a}	3.48^{b}	2.51^{a}	4.30^{c}	2.47^{a}	4.46^{c}	4.40^{a}	5.52^{a}	8.61^{b}	5.90^{a,b}	7.58^{a,b}	5.23^{a}	7.50^{a,b}

[a]For each phospholipid and each fatty acid: Values (mean) not bearing the same superscript letter are significantly different at $P < 0.05$. If no superscript appears, values are not different; $n = 5$ in each data set; PC, phosphatidylcholine; PI, phosphatidylinositol.

TABLE 4
Fatty Acid Composition of Rat Liver PC, Rat Liver PE and of Rat Liver PS

	PC							PE							PS						
	Start	Reference group		Structured oil		Randomized oil		Start	Reference group		Structured oil		Randomized oil		Start	Reference group		Structured oil		Randomized oil	
Diet	0 wk	1 wk	3 wk	1 wk	3 wk	1 wk	3 wk	0 wk	1 wk	3 wk	1 wk	3 wk	1 wk	3 wk	0 weeks	1 wk	3 wk	1 wk	3 wk	1 wk	3 wk
14:0[a]	1.37^{b}	1.08^{b}	0.60^{a}	1.24^{b}	0.58^{a}	1.19^{b}	0.50^{a}	—	—	—	—	—	—	—	0.59^{a,b}	0.82^{b}	0.46^{a,b}	0.81^{b}	0.22^{a}	0.89^{b}	0.45^{a,b}
16:0	31.98^{c,d}	32.80^{d}	25.52^{a}	31.72^{c,d}	30.08^{b,c}	31.34^{c,d}	28.63^{b}	23.26^{c}	24.87^{d}	21.11^{a,b}	22.99^{c}	20.09^{a}	22.22^{b,c}	19.75^{a}	5.69^{a,b}	6.94^{a,b}	5.23^{a}	8.24^{b}	6.23^{a,b}	7.56^{a,b}	6.68^{a,b}
16:1	0.23^{a,b}	0.11^{a}	0.85^{d}	0.25^{a,b}	0.67^{c,d}	0.18^{a,b}	0.45^{b,c}	0.06^{a}	0.03^{a}	0.24^{c}	0.04^{a}	0.20^{c}	0.03^{a}	0.15^{b}	0.21	0.52	0.22	0.24	0.10	0.23	0.31
18:0	14.33^{b}	13.55^{a,b}	17.27^{c}	14.59^{b}	12.72^{a}	15.00^{b}	14.40^{b}	21.84^{a,b}	21.03^{a}	22.31^{a,b,c}	22.67^{a,b,c}	22.99^{a,b,c}	24.06^{c}	23.35^{b,c}	43.61^{a,b}	43.95^{a,b}	44.14^{a,b}	45.15^{a,b}	43.84^{a,b}	46.04^{b}	42.50^{a}
18:1	4.52^{a,b}	3.55^{a}	8.66^{c}	5.10^{b}	13.15^{d}	5.67^{b}	13.60^{d}	1.59^{a}	0.82^{a}	4.43^{b}	1.77^{a}	5.22^{b}	0.94^{a}	5.51^{b}	5.73^{b}	5.39^{b}	4.29^{a}	6.42^{b}	6.23^{b}	6.04^{b}	5.84^{b}
18:2n-6	7.89^{a,b}	7.97^{a,b}	12.43^{c}	8.98^{b}	8.46^{b}	9.48^{b}	6.67^{a}	2.32^{a,b}	2.13^{a}	5.14^{e}	2.89^{c,d}	3.21^{d}	3.21^{b}	2.61^{b,c}	1.39^{a}	1.38^{a}	2.30^{b}	1.48^{a}	1.20^{a}	1.50^{a}	1.39^{a}
20:1	0.12	0.03	0.07	0.10	0.85	0.07	0.42	—	—	—	—	—	—	—	0.27^{a,b}	0.11^{a}	0.22^{a}	0.29^{a,b}	0.38^{a,b}	0.42^{a,b}	0.55^{b}
20:3n-6	0.87	0.90	0.82	0.93	0.77	1.18	0.84	0.33^{a}	0.33^{a}	0.42^{a,b}	0.40^{a}	0.36^{a}	0.49^{b}	0.34^{a}	1.08^{b,c}	1.06^{b,c}	0.93^{b}	1.05^{b,c}	0.64^{a}	1.14^{c}	0.65^{a}
20:4n-6	21.71^{c}	21.05^{c}	18.30^{b}	18.11^{b}	8.23^{a}	18.24^{b}	7.21^{a}	18.75^{b}	18.31^{b}	21.09^{c}	17.58^{b}	8.10^{a}	17.73^{b}	6.97^{a}	28.07^{c}	25.77^{c}	17.65^{b}	18.14^{b}	4.62^{a}	16.51^{b}	4.84^{a}
20:5n-3	0.15^{a}	0.17^{a}	0.53^{a}	0.30^{a}	6.63^{b}	0.28^{a}	9.00^{c}	0.19^{a}	0.21^{a}	0.64^{a}	0.39^{a}	8.19^{b}	0.48^{a}	9.33^{c}	0.38^{a}	0.55^{a}	0.40^{a}	0.58^{a}	3.14^{b}	0.58^{a}	3.74^{b}
22:4n-6	0.23^{c}	0.18^{b,c}	0.28^{d}	0.21^{b,c}	—^{a}	0.16^{b}	0.01^{a}	0.92^{c}	0.70^{b}	0.84^{b,c}	0.78^{b,c}	0.08^{a}	0.69^{b}	0.07^{a}	1.79^{c}	1.41^{b}	1.24^{b}	1.25^{b}	0.18^{a}	1.28^{b}	0.30^{a}
22:5n-6	0.09^{a}	0.11^{a}	0.52^{b}	0.10^{a}	0.05^{a}	0.11^{a}	0.05^{a}	0.24^{a}	0.19^{a}	0.91^{b}	0.22^{a}	0.07^{a}	0.26^{a}	0.07^{a}	0.16^{a}	0.10^{a}	1.16^{b}	0.08^{a}	0.13^{a}	0.12^{a}	0.19^{a}
22:5n-3	1.38^{a}	1.98^{b}	2.29^{b}	2.41^{b}	1.06^{a}	2.32^{b}	1.21^{a}	2.10^{a}	3.03^{b}	3.98^{c}	3.48^{b,c}	1.75^{a}	3.51^{b,c}	1.96^{a}	1.04^{a}	1.37^{a,b}	2.39^{d,e}	1.71^{b,c}	2.15^{c,d,e}	1.87^{b,c,d}	2.62^{e}
22:6n-3	14.37^{b}	15.76^{b}	9.39^{a}	15.08^{b}	15.62^{b}	13.88^{b}	15.86^{b}	27.55^{b,c}	27.60^{b,c}	17.07^{a}	25.92^{b,c}	28.60^{c}	25.55^{b}	28.770^{c}	9.00^{a}	9.22^{a}	17.59^{c}	13.31^{b}	30.29^{d}	14.60^{b,c}	28.96^{d}

[a]For each phospholipid and each fatty acid: Values (mean) not bearing the same superscript letter are significantly different at $P < 0.05$. If no superscript appears, values are not different; $n = 5$ in each data set. See Tables 2 and 3 for abbreviations.

types of TAG containing similar levels of 22:6n-3, but having different TAG structure, i.e., a specific structured TAG and a randomized TAG containing 56 and 33%, respectively, of the 22:6n-3 in the *sn*-2 position. Both rat milk and human milk contain MCFA as well as short-chain fatty acids (SCFA). The TAG were therefore manufactured from fish oil and butterfat, which are available fats that provide n-3 PUFA and MCFA, respectively. The SCFA and MCFA in the diet of the newborn are important because these fatty acids are readily oxidized and thus provide energy to the growing infant. The interesterified fats had considerably higher levels of PUFA than rat milk and lower levels of SCFA and MCFA. This could affect their degradation by maternal milk lipase (24) available during the night when the pups stayed with their dams as well as by lingual lipase (25). On the other hand this did not result in different body weights in the present experiment. Previous studies have demonstrated that randomized TAG containing MCFA and PUFA are well absorbed (26), but it has also been demonstrated that specific structured TAG may possess superior absorption properties at low intestinal hydrolytic activity (12). In all mammals, the milk has a particular TAG structure with 16:0 located preferentially in the *sn*-2 position, which allows for a high energy uptake (27). In formulas for pre-term infants, this TAG structure may not be optimal if a high uptake of n-3 PUFA is desired since n-3 PUFA are released slowly from the *sn*-1,3 positions of TAG by pancreatic lipase (28).

TABLE 5
Fatty Acid Composition of Rat Adipose Tissue Triacylglycerols

	Start 0 wk	Reference group 3 wk	Structured oil 3 wk	Randomized oil 3 wk
10:0[a]	5.29[b]	2.00[a]	1.71[a]	1.80[a]
12:0	8.77[b]	5.60[a]	4.81[a]	4.30[a]
14:0	9.16	8.07	9.42	8.06
16:0	26.80[a]	27.65[a]	29.76[b]	28.10[a]
16:1n-7	2.96[a]	2.80[a]	5.24[b]	5.08[b]
18:0	3.29[a]	3.49[a]	4.60[b]	4.87[b]
18:1n-9/7	19.73[a]	19.49[a]	26.70[b]	28.26[b]
18:2n-6	16.35[b]	24.62[c]	8.71[a]	9.08[a]
18:3n-6	0.56[b]	0.34[a]	0.18[a]	0.23[a]
18:3n-3	0.82[a]	1.56[b]	0.98[a]	1.04[a]
20:1	0.19[a]	0.49[a]	2.43[b]	2.51[b]
20:3n-6	0.63[b]	0.24[a]	0.14[a]	0.15[a]
20:4n-6	2.11[c]	0.81[b]	0.37[a]	0.43[a]
20:5n-3	0.09[a]	0.20[a]	0.88[b]	1.15[c]
22:5n-3	0.44	0.31	0.28	0.42
22:6n-3	1.23[b]	0.74[a]	1.85[c]	2.32[d]

[a]For each fatty acid: Values (mean) not bearing the same superscript letter are significantly different at $P < 0.05$. If no superscript appears, values are not different; $n = 5$ in each data set.

It was possible to increase the level of 22:6n-3 in brain PS and PC, but not in brain PE and PI compared with the reference group fed solely rat milk. The physiological significance of these changes is not clear. The increase in 22:6n-3 in the brain phospholipids was accompanied by decreases in n-6 PUFA: 20:4n-6, 22:4n-6, and 22:5n-6, which has also been found by others (8,29). These fatty acids decreased in all three groups as a function of time, but to significantly lower levels in the experimental groups than in the reference group. The brain is able to synthesize 22:6n-3 from 18:3n-3 (30), but the majority of the accumulating 22:6n-3 seems to be synthesized in the liver (31) if 18:3n-3 is the main source of n-3 PUFA and transported, possibly by chylomicrons (32), to the brain. If dietary 22:6n-3 is available this probably can be transported to the brain either in chylomicrons or in very low density lipoprotein. In the brain phospholipids no effects of the TAG structure on the deposition of 22:6n-3 could be detected (Tables 2,3). This may reflect that the specific structured TAG did contain some 22:6n-3 in the *sn*-1/3 positions or that in slowly growing tissues like the brain the uptake is independent of TAG structure at least if sufficient n-3 PUFA is available. In contrast to this, we have previously demonstrated that the dietary TAG structure is reflected in the chylomicron TAG structure (33) which again affects the clearance of chylomicrons and short-term deposition in, e.g., the liver and kidney (34).The decrease in 20:4n-6 is of particular interest since 20:4n-6 has an important role in signal transduction (35) and is a precursor for eicosanoids (36). The resistance of PI, phosphatidylinositol, and phosphatidylinositol-bisphosphate to even multigeneration exposure to n-3 PUFA has been described before (10,37). However, a decrease in 20:4n-6 in PI is a general phenomenon from 0 to 3 wk as also found by Bourre *et al.* (5). The changes in fatty acid profile may reflect the onset of brain myelinization during which large saturated and monounsaturated fatty acids accumulate in the myelin sheets (38). In the rat this is initiated in the first week of life, peaks at about 14 d after birth and continues into the fourth week of life (39) and in humans starts before birth and peaks at about five months (40). We find no influence of the dietary fat on the deposition of 18:1 in the brain phospholipids during this process. This again may reflect that the rats were fed diets supplying adequate fatty acids.

The fatty acid composition in the liver phospholipids was, as shown by others (8,41), much more susceptible to influence by dietary fatty acids than the brain. No major differences related to TAG structure were observed. This may result from the relatively high levels of 22:6n-3 in both experimental diets. The experimental fats were, however, able to maintain high hepatic levels of 22:6n-3 while a major reduction in 22:6n-3 from PC and PE in the reference group occurred during this period. The experimental fats resulted also in deposition of 22:6n-3 in PS. In liver PE and PC, the level of 22:6n-3 was nearly constant in the study period in the experimental groups, whereas the level decreased significantly from 1 to 3 wk in the reference group. In liver PS the level of 22:6n-3 increased in all groups as a function of time, but reached significantly higher levels in the experimental groups. In the liver PE and PC are the major phospholipids, contributing approximately 75% of all phospholipids, and the decrease in 22:6n-3 may reflect the transport of synthesized 22:6n-3 to the developing CNS. Since the brain phopholipids are relatively unaffected by the dietary n-3 PUFA and the liver increases the content, we conclude that surplus of n-3

PUFA is deposited in the liver, but it must be noted that this has profound influence on the liver fatty acid profile and may later be mobilized. The effect is, however, solely related to the dietary level of n-3 PUFA and in this experiment not affected by TAG structure. As in the brain, the level of 20:4n-6 in the liver decreased as a function of time and was replaced by 20:5n-3, except in PE where the level in the reference group increased from 1 to 3 wk. This may reflect an export of 20:4n-6 from the liver, and also that the dietary level of 18:2n-6 was lower in the experimental groups than in the control group. The level of 20:4n-6 in plasma and the growth of newborn infants are correlated (41), indicating that dietary 20:4n-6 for newborn infants may be as important as 22:6n-3, which is supported by observed growth retardation correlated with reduced 20:4n-6 contents in red blod cells (42).

The growth of the pups as well as the incorporation of n-3 fatty acids indicates that the fats were well absorbed and with similar efficiency. Significantly higher deposition of n-3 PUFA following intake of the randomized fat in the adipose tissue may result from the activity of lipoprotein lipase which is *sn*-1,3 specific, since the chylomicron TAG structure largely reflects the TAG structure of the dietary fat (34). This may indicate that larger differences in tissue depositions could have resulted if the dietary fats had been more specific in TAG structure. The postnatal demand for long-chain PUFA may be met by dietary supplies, as well as by stores in the newborn formed during the embryonic period by export from the maternal body. In the present experiment, small amounts of both 20:4n-6 and 22:6n-3 were found in the adipose tissue of the newborn rats (2.1 and 1.2%, respectively, at 0 wk, and a total amount of long-chain (C_{20} and C_{22}) PUFA of 5.3%). After three weeks, the level of 22:6n-3 was reduced by almost 50% in the reference group, either by depletion for use elsewhere in the body or by dilution with accumulated fatty acids in the adipose tissue. In the experimental groups, the level of 22:6n-3 was significantly increased in the same period, suggesting that the demand for PUFA from the CNS was met and that surplus PUFA accumulated in the adipose tissue. The level of 22:6n-3 in the intervention diet was 3.8% of total fatty acids which is approximately six times the level in the milk from rats fed ordinary rat chow. At this dietary level, only minor effects of the TAG structure of dietary fat used in this experiement are observed.

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Phospholipid Fatty Acid Composition in Type I and Type II Rat Muscle

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ABSTRACT: The fatty acid composition of the membrane phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine in insulin-sensitive Type I (soleus) and insulin-resistant Type II (EDL) muscle is not known. In the present studies, soleus and EDL muscles were removed from 250–300 g Sprague-Dawley rats, and the fatty acid composition of total and individual phospholipid (PL) species was quantitated. As expected, triglyceride content was increased twofold in soleus muscle. No quantitative differences in the individual PL species or cholesterol content were found between the two muscles. However, a striking difference in PL fatty acid composition was observed in the PC fraction. An increase in 16:0 with decreases in 18:0, 18:1, 22:5n-3, and 22:6n-3 ($P < 0.001$ for each) was observed in the PC fraction of EDL compared to that from soleus, consistent with reduced elongation of PC fatty acids. Inhibition of fatty acid oxidation with the carnitine palmitoyl transferase-I inhibitor, etomoxir, did not alter the fatty acid pattern in either muscle. We conclude that an alteration in PL fatty acid composition consistent with reduced elongation of both saturated and unsaturated fatty acids is observed in Type II muscle. The restriction of these alterations to the PC fraction has important implications.

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Type I and Type II muscle fibers differ in their responsiveness to insulin. Type I muscle (slow-twitch), with its greater number of mitochondria and greater oxidative capacity, is more responsive to insulin than is Type II muscle (fast-twitch) with its greater glycolytic capacity (1–7). Differences in insulin receptor number (8–10) and receptor tyrosine kinase activity (10) have been variably reported, but no *in vitro* study has shown the expected rightward shift in the insulin dose-response curve consistent with these characteristics of Type II muscle. A more attractive explanation for the enhanced responsiveness of Type I muscle is its greater capacity for glucose transporter (Glut-4) recruitment compared to that in Type II muscle (5,7,11). Since phospholipid (PL) composition is known to influence membrane protein incorporation as well as enzyme activity and receptor function (12,13), differences in membrane PL fatty acid composition may play an important role in insulin responsiveness.

The present study examines the PL content of Type I and Type II muscle in the rat. Since the major PL in the outer layer of the sarcolemma bilayer is phosphatidylcholine (PC) (14), whereas phosphatidylethanolamine (PE) is preferentially concentrated in the inner layer, the fatty acid composition of these two predominant PL species (choline, PC and ethanolamine, PE) as well as the quantitatively less significant phosphatidylinositol (PI) in muscle have been emphasized. To our knowledge, no studies to date have examined the fatty acid composition of PC and PE in Type I and Type II muscle. To determine if differences in fatty acid oxidation in Type I and Type II muscle contribute to putative differences in the PL fatty acid composition, the carnitine palmitoyl transferase I (CPT-1) inhibitor, etomoxir, was administered in a separate series of experiments. The studies have demonstrated a striking variation in the fatty acid composition of PC in the two muscle types which was not present in either PE or the other classes of PL.

METHODS

Overnight fasted male Sprague-Dawley rats with a mean weight of 276.1 ± 7.0 g (range 250–300 g) were killed by cervical dislocation after undergoing ether anesthesia. Serum was obtained by cardiac puncture, and soleus and extensor digitorum longus (EDL) muscles were removed for PL analyses. Soleus muscle consists mainly of slow-twitch, Type I (84%) red fibers, and EDL muscle consists mainly of Type II white fibers (38% Type IIA; 59% IIB) (15).

In experiments to examine the effect of inhibition of fatty acid oxidation, etomoxir (a CPT-1 inhibitor) was administered at a dose of 15 mg/kg/d S.Q. for six weeks (16). The muscle PL composition was compared to that of litter mates fed *ad libitum*. Body weights of control and etomoxir-fed animals were measured at the end of the treatment period.

Lipid analysis [PL, triglycerides (TG), and cholesterol]. Soleus and EDL muscles (100–120 mg) were removed from the overnight-fasted male rats. The muscles were homogenized in chloroform and methanol (2:1, vol/vol) containing

[1]Deceased (June 28, 1996).

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Abbreviations: CA, cardiolipin; DAG, diacylglycerol; EDL, extensor digitorum longus; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; TG, triglycerides.

0.01% butylated hydroxytoluene using a Rockwell homogenizer, and total lipids were extracted by the method of Folch *et al.* (17). Serum samples were also treated with chloroform and methanol prior to extraction. To quantitate the various PL species, cholesterol, and TG, lipids from the Folch extraction were separated by high-performance thin-layer chromatography as described by Alvarez and Ludman (18). Following chromatography, the plates were dipped into 10% $CuSO_4$ in 8% H_3PO_4 and oven-charred at 130°C for 30 min. The plates were scanned immediately using an ultrascan XL laser densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden) and quantitated according to Macala *et al.* (19). As the charring method is only semiquantitative, the major PL species (PC and PE) were also determined by the Naphthol Blue Black staining method which has been validated for accuracy (20).

PL fatty acid composition. PL fatty acid composition was analyzed according to Storlien and coworkers (21) with minor modifications. Hemi-muscles (50–60 mg) were homogenized as described above and total lipids were extracted by the method of Folch *et al.* (17). The lipid extracts were dried under nitrogen, dissolved in 10 mL of hexane and applied to 3 mL silica gel columns (J.T. Baker, Inc., Phillipsburg, NJ). After elution of the less polar lipids with 20 mL hexane followed by 10 mL dichloromethane, PL were eluted with 10 mL methanol. The methanol eluates were dried under nitrogen and transmethylated with 1.5 mL 1 N methanolic HCl at 80°C overnight. Fatty acid methyl esters were extracted with 6 mL hexane and dried under nitrogen.

To determine the fatty acid composition of individual PL species, PC, PE, sphingomyelin (SM), PI, cardiolipin (CA), and phosphatidylserine (PS) were first separated by thin-layer chromatography on silica gel G plates (Whatman LK6D, Clifton, NJ) using a solvent system consisting of chloroform/ethanol/triethylamine/water (30:34:30:8, by vol) for the first development and hexane/dimethyl ether (50:50, by vol) for the second development. PL were visualized under ultraviolet (UV) light after spraying the plate with rhodamine G. The separated PL spots were collected by scraping and placed into glass tubes. Fatty acid methyl esters were prepared as described above by treatment with methanolic HCl.

Fatty acid methyl esters from the total PL fractions as well as the individual PL species were redissolved in 20 μL hexane and analyzed on a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) equipped with a 30 m × 0.2 mm fused silica capillary column (Omega wax 320; Supelco, Inc., Bellefonte, PA) and flame-ionization detector. The injection temperature was 250°C and detector temperature was 300°C. The initial oven temperature was 140°C. After five minutes, the oven temperature was increased from 140 to 200°C at a rate of 20°C/min, then to 280°C at 5°C/min. Fatty acids were identified by comparing their retention times with those of authentic standards.

Most of the gas chromatograph peaks were identified as specific fatty acid methyl esters. In the total PL and PC preparations, these fatty acid methyl esters accounted for 87 and 93% of the total integrated area, respectively, whereas they comprised only 79% of the integrated area in the PE fraction. In all samples, however, there were peaks immediately preceding palmitate (16:0) and stearate (18:0), which were suspected to be dimethyl acetal derivatives of fatty aldehydes released from ether PL (plasmalogens). To confirm this identification, PE from bovine brain containing 60% plasmalogens (Sigma Chemical Co., St. Louis, MO) was chromatographed before and after mild acid fume hydrolysis (22). After mild acid fume hydrolysis and separation on thin-layer chromatography plates, the peaks before 16:0 and 18:0 suspected of being derived from plasmalogens completely disappeared, confirming their identity as dimethyl acetals.

PL, TG, cholesterol, and fatty acid standards were obtained from Sigma. High-performance pre-coated silica gel Hp-K plates (10 cm × 10 cm) were purchased from Whatman. All other reagents and solvents were of analytical or HPLC-grade from Sigma or Fisher (Pittsburgh, PA).

RESULTS

Figure 1 displays the lipid composition of soleus and EDL muscles as determined by the charring method after separation by thin-layer chromatography. All six of the PL species were expressed as a percentage of total PL, whereas TG and cholesterol were expressed as a percentage of total lipids, (PL, TG, and cholesterol). There were no significant differences in PL species between the two muscles which collectively accounted for approximately 75% of total tissue lipids. The major intermuscle difference was the twofold greater TG composition in soleus muscle. Because the charring method is only semiquantitative, the two major PL species (PC and PE) were quantitated by the more accurate Naphthol Blue Black staining method. Using this technique, the concentration of PC was greater than that of PE, but no significant difference in the PC/PE ratios was noted in the two muscles (PC/PE ratio 1.41 ± 0.07 in soleus and 1.46 ± 0.07 in EDL).

The fatty acid composition of total PL and the individual PL classes was determined by gas chromatography. Approximately 90% of the chromatographic peaks were identified as

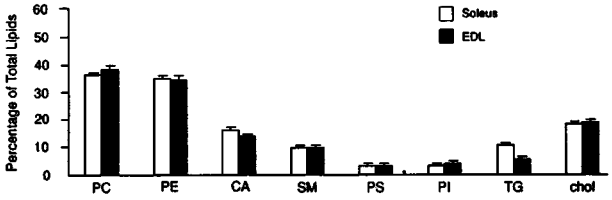

FIG. 1. Lipid composition of soleus and extensor digitorum longus (EDL) muscles. Phospholipid species are expressed as a percentage of total phospholipids, whereas triglycerides and cholesterol are expressed as a percentage of total lipids (including all phospholipids); PC, phosphatidylcholine; PE, phosphatidylethanolamine; CA, cardiolipin; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; TG, triglyceride; CHOL, cholesterol. Means ± SEM shown. $n = 12$ animals. Triglycerides are significantly increased in soleus ($P < 0.001$).

specific fatty acid methyl esters or dimethyl acetal derivatives of plasmalogen PL. Two major dimethyl acetal peaks (16:0 dimethyl acetal and 18:0 dimethyl acetal) derived from ether phospholipids (plasmalogens) were observed predominantly in the chromatograms of PE. These peaks accounted for 10.02 ± 0.28% (soleus) and 9.03 ± 0.35% (EDL) of the total methylated derivatives (fatty acids + aldehydes) on the chromatograms of PE. In contrast, these same peaks contributed only 0.69 ± 0.13% (soleus), and 0.66 ± 0.08% (EDL) of the peak area on chromatograms of PC. The plasmalogen content of PE is known to be greater than that of PC (23). These dimethyl acetal derivatives were quantitatively similar in the two muscles.

The fatty acid composition of the total PL fractions from the two muscles as well as from rat serum are shown in Table 1. The major differences in PL fatty acid composition between the two muscles were an increase in 16:0 and a decrease in 18:0 in Type II (EDL) muscle. The very different PL fatty acid composition of muscle compared to serum is indicative of local membrane remodeling (24). For example, the percentage of docosahexanoic acid (22:6n-3) was five times greater in muscle PL than in serum, whereas the percentage of oleic acid (18:1n-9) was almost twofold greater in serum.

The fatty acid compositions of individual PL classes are shown in Table 2. The increased palmitate in PC and increased stearate in PE are consistent with the known predilection of PC- and PE-synthesizing enzymes for diacylglycerol (DAG) with palmitate or stearate in the *sn*-1 position, respectively (25). The PE-synthesizing enzymes also favor synthesis from DAG with docosahexanoic acid (22:6n-3) in the *sn*-2 position (25), thus explaining the two- to threefold increase in this fatty acid in PE compared to that in PC.

The greatest difference in PL fatty acid content between the two muscles was observed in the saturated fatty acids of the PC species (Table 2). Choline PL from EDL muscle had a much higher 16:0 composition, and a significantly lower 18:0 composition than that from soleus muscle. This was reflected by a PC palmitate (16:0) to stearate (18:0) ratio of 3:1 in EDL compared to the 1:1 ratio in soleus (Fig. 2). Importantly, this ratio was minimally altered by muscle fiber type in the other PL classes (PE, CA, and SM). The longer chain saturated fatty acids (19:0, 20:0, 22:0, 24:0) of PC were also reduced in the EDL compared to the soleus, although the difference was significant only for 19:0. As shown in Table 2, oleic acid (18:1), an elongation as well as desaturation product of palmitate, was reduced in EDL, whereas linoleic (18:2n-6) and linolenic (18:3n-6) acids, two essential fatty acids, were not significantly different in the two muscles. However, the n-3 elongation products 22:6n-3 and docosapentaenoic acid (22:5n-3) were significantly reduced in the PC fraction of EDL muscle.

In contrast to PC, the differences in fatty acid composition of the PE fractions between the two muscle types were relatively trivial (Table 2). As shown in Figure 2, the 16:0 to 18:0 ratio in PE was slightly but significantly increased in EDL compared to soleus (0.16 ± 0.01 to 0.19 ± 0.01, $P < 0.003$). The ratio of 16:0 to 18:0 in the SM and CL fractions was almost identical between the two muscles (Fig. 2). Thus, the minimal intermuscle differences in fatty acid composition of these other PL classes reduced the magnitude of the difference detected when the fatty acid composition from total PL was compared in the two muscles. In fact, in the case of 22:6n-3, the significant reduction of this fatty acid in PC of EDL (Table 2) was effectively masked in the fatty acid analysis of the total PL fraction by the increase in PE 22:6n-3 in EDL (Tables 1 and 2).

Because of the putative role of PI in muscle signaling, the fatty acid composition of this species is shown (Table 3), despite its low content in rat muscle PL (2.5%). Stearate was the predominant saturated fatty acid in PI, being higher than in any of the other species. Stearate was significantly higher ($P < 0.002$) in the EDL and the 16:0 to 18:0 ratio (0.048 ± 0.006 vs 0.039 ± 0.004) was slightly but significantly lower in the EDL (opposite to the ratio observed in PC). No differences in arachadonate (20:4n-6) content in PI were found be-

TABLE 1
Phospholipid Fatty Acid Composition in Serum and Muscle

Fatty acid (%)	Serum PL	Soleus PL	EDL PL
14:0	0.93 ± 0.11[a]	0.37 ± 0.03	0.34 ± 0.04
15:0	0.46 ± 0.02[a]	0.27 ± 0.03	0.27 ± 0.02
16:0	19.21 ± 0.09[a]	11.34 ± 0.31[b]	16.90 ± 0.39
17:0	0.59 ± 0.03	0.54 ± 0.02	0.55 ± 0.01
18:0	20.90 ± 1.00[c]	19.09 ± 0.25[b]	16.15 ± 0.22
16:1n-9	1.46 ± 0.20	0.52 ± 0.02	0.53 ± 0.03
18:1n-9	10.52 ± 0.62[a]	6.89 ± 0.15[b]	5.68 ± 0.16
18:2n-6	14.71 ± 0.79	16.59 ± 0.29	16.33 ± 0.39
18:3n-6	0.60 ± 0.06[a]	0.22 ± 0.01[b]	0.15 ± 0.01
20:4n-6	12.25 ± 0.50	11.74 ± 0.22	12.12 ± 0.26
22:4n-6	0.27 ± 0.03[a]	0.59 ± 0.03[b]	0.51 ± 0.02
22:5n-3	0.69 ± 0.03	3.06 ± 0.09	2.82 ± 0.09
22:6n-3	2.74 ± 0.18[a]	13.73 ± 0.48	12.83 ± 0.51

[a] $P < 0.01$, seum vs. soleus and extensor digitorum longus (EDL).
[b] $P < 0.001$, soleus vs. EDL.
[c] $P < 0.01$, serum vs. EDL alone; $n = 12$ for soleus and EDL: $n = 6$ for serum from overnight-fasted rats. Phospholipid (PL) fatty acids accounting for less than 0.5% of the total are not shown.

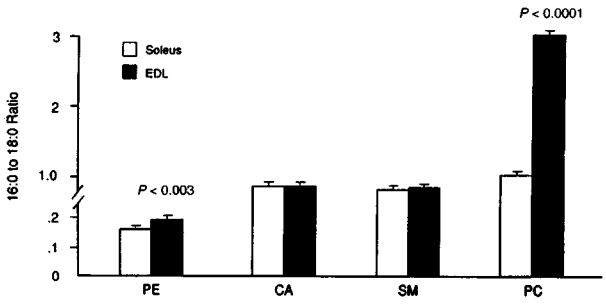

FIG. 2. Fatty acid "elongation ratio" (16:0 to 18:0) in phospholipid species from soleus and EDL muscles. See Figure 1 for abbreviations. Data are expressed as means ± SE; $n = 12$ for PC and PE, $n = 4$ for CA and SM.

TABLE 2
Phospholipid Fatty Acid Composition of Phosphatidylcholine and Phosphatidylethanolamine from Soleus and EDL Muscles

Fatty acid	Phosphatidylcholine		Phosphatidylethanolamine	
(%)	Soleus	EDL	Soleus	EDL
16:0	16.96 ± 0.72[a]	27.09 ± 0.84	3.52 ± 0.13[a]	4.35 ± 0.20
17:0	0.58 ± 0.01[a]	0.66 ± 0.02	0.55 ± 0.07	0.38 ± 0.02
18:0	16.67. ± 0.44[a]	8.83 ± 0.27	22.55 ± 1.02	22.39 ± 0.81
16:1n-9	0.95 ± 0.15	1.36 ± 0.18	0.24 ± 0.05	0.28 ± 0.02
18:1n-9	9.74 ± 0.30[a]	6.89 ± 0.27	4.13 ± 0.29	4.07 ± 0.23
18:2n-6	16.12 ± 0.34	18.15 ± 0.56	5.00 ± 0.13[a]	6.06 ± 0.15
20:4n-6	14.31 ± 0.21	14.71 ± 0.68	10.93 ± 0.29[a]	7.98 ± 0.14
20:5n-3	0.78 ± 0.15	0.77 ± 0.06	0.97 ± 0.21	0.80 ± 0.11
22:4n-6	0.35 ± 0.03	0.25 ± 0.02	0.62 ± 0.06	0.62 ± 0.05
22:5n-3	3.69 ± 0.18[a]	2.49 ± 0.09	3.97 ± 0.17	4.15 ± 0.13
22:6n-3	11.74 ± 0.60[a]	8.28 ± 0.44	25.19 ± 0.52	27.32 ± 0.59
24:1n-9	0.33 ± 0.05	0.23 ± 0.05	0.45 ± 0.08	0.47 ± 0.07

[a]$P < 0.001$, soleus compared to EDL.Phospholipid fatty acids accounting for less than 0.5 % of the total are not shown; $n = 12$ for overnight-fasted rats. See Table 1 for abbreviation.

tween soleus and EDL muscles. However, the content of 18:2n-6 was significantly increased in soleus compared to EDL (Table 3)

To determine whether differences in fatty acid oxidation rates of the two muscles might be responsible for the alteration in PC fatty acid content, the fatty acid composition of muscle PC and PE from rats treated for six weeks with the CPT-1 inhibitor etomoxir was compared to that of untreated animals. In Figure 3, it can be observed that the 16:0/18:0 ratio of PC was not altered in etomoxir-treated animals. Thus, greater rates of fatty acid oxidation in Type I (soleus) compared to Type II (EDL) muscle are unlikely to explain the divergent 16:0 to 18:0 ratios in PC. Although the mean weights of control animals and etomoxir-treated animals were not significantly different, the etomoxir-treated animals exhibited cardiac hypertrophy (0.0029 ± 0.0003 vs. 0.0025 ± 0.0002 heart/body weight ratio, $P < 0.005$) and visibly fatty livers corroborated by marked increase in TG content as assessed by thin-layer chromatography.

DISCUSSION

Since membrane PL may play a role in signal transduction (26), as well as influence membrane fluidity affecting membrane enzyme and receptor activity (12,13), we characterized the PL in membranes of the two muscle types disparate for insulin responsiveness (soleus, Type I and EDL,Type II). PL were determined on total cell lipid extracts rather than on isolated membrane fractions. However, it is generally considered that the measurement of total cell PL reflects that of cell membranes, and that the ranking of the major PL fatty acids is preserved among the various cell membranes (27). No differences in the PL species composition of soleus and EDL muscles were noted.

Analysis of the total PL fatty acid composition was not so informative as the fatty acid composition of the individual PL species. Since PC and PE contributed over 70% of the total PL, the major focus was on the analysis of the fatty acid composition in these two classes. Striking differences in the fatty acid composition of soleus and EDL were observed in the PC

TABLE 3
Fatty Acid Composition of Phosphatidylinositol from Soleus and EDL Muscles[a]

Fatty acid	Saturated fatty acids		
(%)	Soleus	EDL	*P* value
16:0	1.67 ± 0.19	1.46 ± 0.12	
18:0	35.16 ± 0.58	37.80 ± 1.06	<.05
	Unsaturated fatty acids		
18:1n-9	3.61 ± 0.013	2.99 ± 0.51	
18:2n-6	2.82 ± 0.18	1.96 ± 0.14	<.001
20:4n-6	27.62 ± 1.12	25.74 ± 0.62	
22:4n-6	2.83 ± 0.20	2.57 ± 0.17	
22:5n-3	4.48 ± 0.17	4.9 2 ± 0.19	
22:6n-3	9.77 ± 0.35	10.56 ± 0.23	

[a]Because of low phosphatidylinositol (PI) content, PI was pooled from each pair of muscles from four animals. Fatty acids accounting for more than 1% of total PI fatty acids are shown, $n = 4$. See Table 1 for other abbreviation.

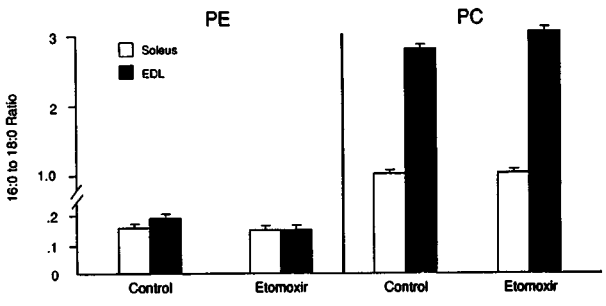

FIG. 3. Fatty acid "elongation ratio" (16:0 to 18:0) in phosphatidylethanolamine and phosphatidylcholine fractions from soleus and EDL muscles removed from control and etomoxir-treated rats. Data are expressed as means ± SEM. Ratio is significantly elevated in PC of EDL muscle from both control and etomoxir-treated animals ($P < 0.0001$); $n = 12$ overnight-fasted animals. See Figure 1 for abbreviations.

fraction. However, because of minor intermuscle differences in fatty acids from the other PL classes (PE as well as SM, CL, and PI), the dramatic differences in 16:0 and 18:0 seen in PC were partially masked when the fatty acid profiles of total PL were analyzed. In addition, the reduction in 22:6n-3 observed in the PC fraction from EDL was obscured in the total PL measurements by the increase in this fatty acid in PE.

Major differences in the fatty acid composition of PC were observed between soleus and EDL muscles. In addition to the relative increase in 16:0 and the decrease in 18:0 in EDL, oleic acid (18:1), a desaturase product of 18:0, was also reduced in EDL PC. Linoleic (18:2n-6) and linolenic (18:3n-6) acids, essential fatty acids not synthesized *in vivo*, were not decreased. Elongation products of n-3 fatty acids (22:5n-3 and 22:6n-3) were also decreased in the PC fraction from EDL compared to soleus. However, this was not the case for elongation products of n-6 fatty acids such as 20:4n-6. Since 22:6n-3 inhibits elongation of n-6 fatty acids (28), the failure to demonstrate a reduction in 20:4n-6 in EDL may be secondary to a reduced content of 22:6n-3 and loss of its inhibitory effect on elongation of n-6 fatty acids. The reciprocal changes in 22:6n-3 in EDL PL (decreased in PC and increased in PE) initially raised the possibility of reduced methylation in Type II muscles. In some tissues as much as 40% of PC is formed by methylation of PE, and PE containing 22:6n-3 is a favored substrate for this reaction. However, evidence suggests that synthesis of PC by methylation of PE occurs only in the liver and not in muscle (29).

Although reduced elongation of fatty acids destined for PL in EDL seems a reasonable explanation for the changes in fatty acid profile of the two muscles, it is not clear why such a reduction should be observed in the PC fraction only. We first considered the possibility that the lower rates of fatty acid oxidation in EDL muscle might result in preferential oxidation of elongation products of 16:0 prior to incorporation into membrane PL. However, results from experiments using the CPT-I inhibitor, etomoxir, suggest that this is not the case. Despite clear evidence for an inhibitory effect of etomoxir on fatty acid oxidation, we observed no change in the PL fatty acid profile of the two muscles compared to untreated control animals. Other possibilities for the altered fatty acid profile include differences in substrate specificity for PC biosynthesis or degradation (30). The known preference of the PC-synthesizing enzymes for substrate DAG with 16:0 in the *sn*-1 position (25) could be even more enhanced in Type II muscle such that proportionally less 18:0 is incorporated, providing a profile consistent with reduced elongation activity. This possibility not withstanding, the generalized reduction in elongation products observed in the present studies is more consistent with a global reduction in fatty acid elongation in the PC species obtained from Type II muscle rather than differential multiple substrate specificities for biosynthesis or degradation in the two muscles.

The restriction of intermuscle variations in fatty acid composition to the PC class of PL has important implications for other studies. Borkman and coworkers (31), in their important studies on PL fatty acid composition in man, may not have detected a decreased elongation profile in insulin-insensitive muscle because the entire PL fraction, rather than just PC, was used for analysis. Even so, the negative correlation of palmitate and the positive correlation of stearate with insulin sensitivity in their transformed data suggests the possibility that in man, insulin sensitivity correlates negatively with the PL fatty acid elongation profile (16:0 to 18:0 ratio). These same authors, in conjunction with scientists working with the Pima Indians, have recently reported an decrease in elongase activity in obese, insulin-resistant, Pima Indians (32) which supports this contention.

ACKNOWLEDGMENTS

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Conjugated Linoleic Acid Modulates Hepatic Lipid Composition in Mice

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ABSTRACT: Conjugated linoleic acid (CLA) is a chemoprotective fatty acid that inhibits mammary, colon, forestomach, and skin carcinogenesis in experimental animals. We hypothesize that the ubiquitous chemoprotective actions of dietary CLA in extrahepatic tissues are dependent upon its role in modulating fatty acid composition and metabolism in liver, the major organ for lipid metabolism. This study begins to evaluate the role of CLA in lipid metabolism by determining the modulation of fatty acid composition by CLA. Female SENCAR mice were fed semipurified diets containing 0.0% (Diet A), 0.5% (Diet B), 1.0% (Diet C), or 1.5% (Diet D) CLA (by weight) for six weeks. Mice fed Diets B, C, and D exhibited lower body weights and elevated amounts of extractable total lipid in livers compared with mice fed diets without CLA (Diet A). Analyses of the fatty acid composition of liver by gas chromatography revealed that dietary CLA was incorporated into neutral and phospholipids at the expense of linoleate in Diets B, C, and D; oleate increased and arachidonate decreased in neutral lipids of CLA diet groups. In addition, increasing dietary CLA was associated with reduced linoleate in hepatic phospholipids. In an *in vitro* assay, CLA was desaturated to an unidentified 18:3 product to a similar extent as linoleate conversion to γ-linolenate (9.88, and 13.63%, respectively). These data suggest that CLA may affect metabolic interconversion of fatty acids in liver that may ultimately result in modified fatty acid composition and arachidonate-derived eicosanoid production in extrahepatic tissues. In addition to determining how dietary CLA modulates eicosanoid synthesis, further work is needed to identify enzymatic products that may result from desaturation of CLA.

Lipids 32, 199–204 (1997).

Conjugated linoleic acid (CLA) refers to a group of octadecadienoate (18:2) -derived isomers that are protective against tumorigenesis in several tissues in experimental animals (reviewed in Ref. 1). In particular, CLA inhibits chemically-induced forestomach neoplasia and skin tumor initiation and promotion in mice as well as mammary and colon carcinogenesis in rats (1–5).

CLA is derived from linoleic acid (18:2; *cis*9, *cis*12) and has been identified in cooked meats such as fried ground beef and pork as well as heat-processed dairy products such as cheeses and pasteurized milk and dairy foods (6). Studies designed to determine the chemoprotective effects of CLA have used a synthetic preparation containing several positional and geometric isomers with double bonds predominantly at 9 and 11 or 10 and 12 carbons and various combinations of *cis* and *trans* configurations at each double bond. While CLA constitutes a relatively minor component of the total fatty acid composition of foods, the chemoprotective properties of CLA have been associated with very low levels of CLA in the diet (as low as 0.05% by weight; Ref. 7) either as free or triglyceride esterified fatty acid (3). The relatively low levels of CLA that effectively inhibit carcinogenesis are in contrast to the higher levels of n-6 and n-3 (>5.0% by weight of diet) polyunsaturated fatty acids (PUFA) shown to modulate carcinogenesis (reviewed in Ref. 1).

In early studies, chemoprotection by CLA was associated with incorporation into rat mammary tumor and mouse forestomach phospholipids (8,9). This may be significant in the development of cancer since phospholipid-associated PUFA and their biologically active eicosanoid products mediate several tumor-related events in mammary gland (10), colon (11) and skin (12). The impact of dietary CLA on the relative composition and interconversion of nonconjugated fatty acids (i.e., saturated, and n-3 and n-6 unsaturated) in neutral and phospholipids in liver has not yet been investigated. Furthermore, while desaturated metabolites of CLA may play a role in chemoprevention (13), the relative ability of CLA vs. linoleate to be a substrate for the rate limiting step in linoleate conversion to arachidonate (Δ6 desaturase) has not been definitively established. Because of the interactive effects of n-6 and n-3 PUFA on modulating cancer in extrahepatic tissues, the role of CLA in carcinogenesis may be dependent on altering the metabolism of fatty acids such as linoleate and arachidonate.

The lack of understanding of the physiological impact of CLA on tissue fatty acid composition and metabolism *in vivo* has impeded the elucidation of a mechanism(s) for CLA chemoprevention. Therefore the objectives of the present study were to: (i) evaluate the effects of increasing levels of dietary CLA (0.5–1.5% by weight of diet) on the composition of neutral and phospholipids of mouse liver, the major site of lipid metabolism; and (ii) determine whether CLA is able to act as a substrate for Δ6 desaturase in a manner similar to

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Abbreviations: CLA, conjugated linoleic acid; FAME, fatty acid methyl ester; GC, gas–liquid chromatography; PUFA, polyunsaturated fatty acid.

linoleate in order to gain a better understanding of how CLA may affect the metabolic interconversion of fatty acids [i.e., linoleate conversion to its more desaturated, elongated product, arachidonate (20:4)].

MATERIALS AND METHODS

Materials. The [1-^{14}C]-linoleic acid (50 mCi/mmol: >98.8% radiochemical purity) was purchased from American Radiolabel Company (St. Louis, MO) and [1-^{14}C]-CLA (50 mCi/mmol: >98% radiochemical purity) was custom-synthesized from a portion of radiolabeled parent compound according to the method of Ip *et al.* (8) by American Radiolabel Company. Unlabeled linoleate and CLA (free fatty acids and methyl esters) as well as γ-linolenate methyl ester were purchased from Nu-Chek-Prep (Elysian, MN). Phenylmethylsulfonyl fluoride and ATP were obtained from Sigma Chemical (St. Louis, MO), and solid-phase silica Prep-Sep™ columns were purchased from Fisher Scientific (Fairlawn, NJ). Authentic fatty acid standards were obtained from Sigma Chemical, Supelco (Bellefonte, PA) and Matreya Inc. (Pleasant Gap, PA), and bicinchoninic acid assay reagent was purchased from Pierce Chemicals (Rockford, IL). Diet components were obtained from Dyets, Inc. (Bethlehem, PA). All solvents and other chemicals were of the highest grade commercially available.

Diets and animals. Four experimental diets based on the AIN-76 formulation (14) containing various levels of CLA (Diet A, 0.0%; Diet B, 0.5%; Diet C, 1.0%; or Diet D, 1.5% by weight of diet) shown to modulate mammary tumorigenesis and mouse skin tumor promotion (2) were used in these studies. Various levels of CLA were added to a 5.0% corn oil diet at the expense of dextrose and the fatty acid composition of dietary oils is shown in Table 1. The approximate isomeric composition of CLA (96% pure) reported by Nu-Chek-Prep was 43% *c*9,*t*11- and *t*9,*c*11-CLA, 45% *t*10,*c*12-CLA, 6% *c*9,*c*11-, *c*10,*c*12-, *t*9,*t*11-, *t*10,*t*12-CLA, 2% linoleate and 4% unidentified compound. Diets were prepared on the premises and stored (−20°C) under nitrogen atmosphere in airtight containers for no longer than 2 wk. Diets were fed every other day in clean powder feeders with stainless-steel grids and mice (n = 12 per diet group; 42 d of age; Harlan Sprague-Dawley, Indianapolis, IN) were allowed free access to food and water. Body weights were measured weekly and average food consumption estimated by measuring differences in weight of freshly supplied diet and diet remaining in feeders two days later. After maintaining mice on experimental diets for six weeks, animals were euthanized and livers isolated and frozen in liquid nitrogen. Tissue was stored at −80°C until analyses were performed.

TABLE 1
Fatty Acid Composition of Experimental Diets[a]

	Diet group			
Fatty acids	A	B	C	D
14:0	0.03	0.02	0.02	0.02
16:0	10.10	8.64	8.25	7.15
16:1; 9	0.10	0.09	0.09	0.08
18:0	1.87	1.61	1.54	1.34
18:1; 9	25.01	21.37	20.88	18.16
18:1; 11	0.51	0.49	n.d.	n.d.
18:2; 9, 12 (LA)	56.9	48.92	46.82	40.88
18:2; 9,11 (9,11 CLA)	0.08	6.08	7.68	11.98
18:2;10,12 (10,12 CLA)	0.09	6.45	8.14	12.64
20:1; 11	0.28	0.20	0.19	0.17
20:4; 5,8,11,14	n.d.	0.02	0.02	0.01
UNFA[b]	4.99	6.11	6.37	7.57
P/S	6.92	8.14	8.54	9.86
CLA/LA	0.17	0.26	0.34	0.60

[a]Combinations of oils present in experimental diets were methylated and quantitated using gas chromatography as described in the Materials and Methods section. Results are expressed as a percentage of total fatty acids.
[b]UNFA, unidentified fatty acid; CLA, conjugated linoleic acid; LA, linoleic acid; n.d., not detected; P/S, polyunsaturated/saturated ratio.

Lipid extraction and fatty acid analyses. Total lipids were extracted from liver homogenates by the method of Bligh and Dyer (15). The silica Sep Pack column procedure described by Hamilton and Comai (16) was used to separate phospholipids and neutral lipids. Fatty acid methyl esters (FAME) of neutral and phospholipid fractions were prepared by incubating extracts with tetramethylguanidine at 95°C (17) and quantified by gas-liquid chromatography using a 30-m Omegawax 320 capillary column (Supelco Chromatography Products). Helium flow rate was 30 mL/min, and oven temperature was programmed to start at 175°C for 4 min and increase to 220°C at a rate of 3°C/min. Identification of FAME was accomplished by comparing retention times of FAME to retention times of pure standards spiked with CLA methyl ester preparations. FAME were quantified by determining areas under identified peaks (ChemStation Software; Packard Instrument Company, Meriden, CT).

Δ6 Desaturase activity. In order to determine whether CLA is able to act as a substrate for Δ6 desaturase, the percentage conversion of [1-^{14}C]-linoleate or [1-^{14}C]-CLA to [1-^{14}C]-γ-linolenate or [1-^{14}C]-conjugated 18:3, respectively, in liver microsomes of mice fed chow diets was determined. Microsomes were isolated from liver homogenates in 0.05 M potassium phosphate buffer containing 0.25 M sucrose (pH 7.0), with 100 mM phenylmethylsulfonyl fluoride (18). The microsome-containing pellet was resuspended in ice-cold 0.25 sucrose buffer containing 0.15 KCl (pH 7.2), and protein concentrations were determined using bicinchoninic acid reagent. Because 2.0 mg protein was previously determined to support maximal enzymatic conversion of linoleate to γ-linolenate under identical assay conditions (18), this protein concentration was used in the present studies. A duplicate set of each sample was boiled for 30 min to destroy desaturase activity for determination of nonenzymatc conversion of 18:2 to 18:3. Radiolabeled linoleate or CLA was diluted with unlabeled fatty acid to a specific activity of 6.6 mCi/mmol and incubated with the microsomal protein in potassium phosphate buffer (80 μM; pH 7.2) containing 6.67 μmol ATP, 0.13 μmol coenzyme A, 1.20 μmol nicotinamide adenine dinucleotide, 10.00 μmol $MgCl_2$, 3.00 μmol glutathione, and 83.30 μmol potassium fluoride at 37°C for 25 min (18). The reaction was stopped by the addition of chloroform/methanol (2:1, vol/vol). Fatty acid extracts were methylated, and 18:2 and 18:3 products were separated by reverse-phase thin-layer chromatography

(Analtech, Newark, DE) using acetonitrile/water solvent (90:10, vol/vol) at 40°C. Radiolabeled FAME which co-migrated with authentic standards for 18:2 (linoleate and CLA methyl esters), and 18:3 (γ-linolenate) methyl esters were identified by staining with iodine vapor and autoradiography. Bands were scraped into vials and counted by liquid scintillation. Results of Δ6 desaturase activity were expressed as percentage conversion of [1-^{14}C]-18:2 to [1-^{14}C]-18:3 where enzymatic conversion was quantified by determining the difference between the average total conversion minus nonenzymatic (boiled sample) conversion (18).

To date, metabolites of CLA have not yet been identified so that thin-layer chromatography analyses of CLA metabolites were not compared to an authentic conjugated triene standard. Because [1-^{14}C]-CLA produced a discrete band in reactions containing viable enzyme and this band co-migrated with the triene, γ-linolenate, the unidentified band is assumed to be a triene product of CLA.

Statistical analysis. Data from these studies were analyzed by analysis of variance (General Linear Model, Tukey) using Statistical Analysis System (SAS; Cary, NC).

RESULTS

Body weights and food intake. Body weights were significantly lower ($P < 0.05$; Table 2) in mice fed Diets C and D throughout the six-week feeding period; however, average food intake as measured by food disappearance was similar among the diet groups (Table 3). Due to the lower amounts of weight gain in animals fed higher levels of dietary CLA, the feed/weight gain ratio (feed efficiency) was increased between 1.5- and 2.0-fold in mice fed 0.5 –1.5% dietary CLA (Diets B, C, and D) compared to those fed diets without CLA (Diet A).

Lipid composition of liver. When liver lipids were extracted, a positive association between increasing amounts of dietary CLA and total lipid extracted per gram liver tissue was observed. Livers of mice fed Diets C and D contained approximately twice the amounts of lipid compared to mice fed Diets A or B ($P < 0.05$; Fig. 1). Increasing levels of dietary CLA exhibited dose-responsive effects on the content of several fatty acids in neutral lipids (Table 4); CLA (9,11 and 10,12) was significantly elevated ($P < 0.05$), and linoleate and arachidonate were significantly reduced ($P < 0.05$) in mice fed Diets C and D compared with mice fed diets with lower (Diet B) or no (Diet A) CLA. In addition, the monounsaturated fatty acid, oleate (18:1), was significantly elevated in hepatic neutral lipids of mice fed Diet D compared to mice fed Diets A, B, or C. The total lipids of livers from mice exhibited similar trends to neutral lipids (data not shown). Liver phospholipids of mice fed Diet D contained significantly elevated 9,11-CLA and reduced linoleate compared with mice fed Diets A, B, or C.

Ability of CLA to act as a substrate for Δ6 desaturase. Enzymatic conversion of [1-^{14}C]-linoleate or [1-^{14}C]-CLA to 18:3 products by Δ6 desaturase was similar. The percentage conversion 18:2 to 18:3 was 13.30 ± 2.50 (SD) and 11.78 ± 2.16 (SD) for linoleate and CLA, respectively, ($n = 4$ for linoleate, $n = 5$ for CLA assayed in triplicate). These data suggest that Δ6 desaturase does not utilize either CLA or linoleate preferentially as a substrate; however, competition between these two fatty acids for the enzyme was not determined in the present studies.

DISCUSSION

Numerous studies have shown a correlation between dietary fatty acid-mediated reduction of arachidonate-derived eicosanoids and carcinogenesis (10–12). Because we and others (9,19) have shown that CLA is readily incorporated into cell membrane phospholipids, it is plausible that CLA may inhibit carcinogenesis in extrahepatic tissues by modulating fatty acid composition and subsequent eicosanoid metabolism (reviewed in Ref. 1).

Although biological roles of eicosanoids vary widely, eicosanoids mediate several different processes required for tumorigenesis including cell proliferation (i.e., induction of ornithine decarboxylase and DNA synthesis) and inflammation (i.e., local and systemic immune responses). The fatty acid, arachidonate, can be metabolized to form eicosanoids after being released from phospholipids *via* activation of phospholipase A_2. Arachidonate-derived eicosanoids known to modulate carcinogenesis in the mammary gland and skin include prostaglandin E_2, prostaglandin $F_{2\alpha}$, and prostaglandin D_2 *via* the cyclooxygenase pathway and several products from the lipoxygenase pathway, i.e., hydroxyeicosatetraenoates and leukotrienes. Although each eicosanoid has been shown to exhibit slightly different modulatory effects on tumorigenesis, prostaglandin E_2 production is most often associated with the enhancement of tumor induction. Inhibition of prostaglandin E_2 synthesis by indomethacin in both the mammary gland and

TABLE 2
Effect of Experimental Diets on Body Weight[a]

Diet	Body weights (g)[b]				
	Week 2	Week 3	Week 4	Week 5	Week 6
A	28.20 ± 1.06^{c,d}	31.86 ± 1.70^{d}	33.62 ± 1.57^{d}	35.30 ± 2.00^{d}	37.80 ± 2.47^{d}
B	27.14 ± 0.64^{d,e}	30.16 ± 0.76^{d,e}	31.42 ± 0.83^{d,e}	32.80 ± 1.15^{d,e}	34.86 ± 1.20^{d,e}
C	25.98 ± 1.11^{e}	28.84 ± 0.97^{e}	30.46 ± 1.05^{e}	31.78 ± 2.32^{d,e}	32.90 ± 0.91^{e}
D	25.86 ± 1.72^{e}	28.30 ± 1.79^{e}	29.24 ± 1.65^{e}	31.50 ± 1.92^{e}	32.46 ± 2.25^{e}

[a]At the commencement of the study, mice were weighed and randomly assigned to a diet group. Initial weights of mice were 25.78 ± 3.22 g with no significant difference between diet groups. Mice ($n = 12$ per diet) were fed assigned experimental diets for six weeks.
[b]Values represent means ± SD of body weights (g). Values within each column with different subscripts $^{c, d, e}$ are significantly different ($P < 0.05$).

TABLE 3
Effect of Experimental Diets on Food Disappearance, Body Weight Gain, and Feed Efficiency in Mice

Diet	Food disappearance (g)[a]	Average weight gain (g)[b]	Feed efficiency[c]
A	7.056 ± 2.326	12.02	0.587
B	7.913 ± 1.773	9.08	0.872
C	7.068 ± 1.820	7.12	0.993
D	8.393 ± 2.395	6.68	1.256

[a]Food disappearance was measured during two two-week intervals during the study. Values represent combined means ± SD of food disappearance (g) per mouse per day. There was no significant difference for food disappearance between diet groups.
[b]Average weight gain per diet group was calculated as follows: final average body weight for each diet group – the initial body weight of animals at the commencement of the study (25.78 g).
[c]Feed effeciency = food disappearance/average weight gain.

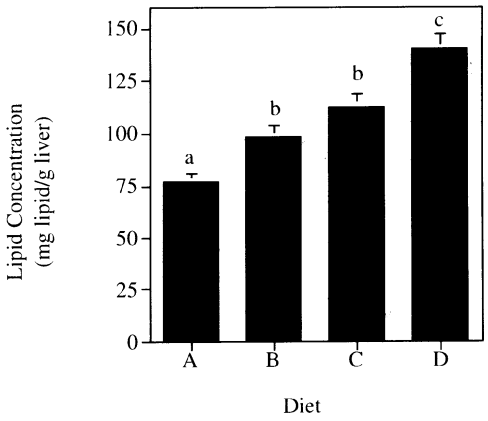

FIG. 1. Effect of dietary conjugated linoleic acid (CLA) on lipid concentration of liver. Mice were fed one of four diets containing various levels of CLA for six weeks. Values represent mean ± SD (n = 4–6 samples). Different superscripts denote significantly different ($P < 0.05$) values for lipid accumulation.

skin has correlated with reduced tumorigenesis in each of these organs (reviewed in Ref. 12). Dietary-induced modifications in phospholipid fatty acid composition where arachidonate is partially replaced with other fatty acids, i.e., n-3 PUFA or linoleate, have correlated with reduction of prostaglandin E_2 (10,20,21). In the case of the mammary gland and pancreas, inhibition of tumor formation was also observed (10,22).

Based on our findings that CLA is a suitable substrate for Δ6 desaturase, membrane-bound CLA and its desaturated and/or elongated products are likely to compete with other PUFA for phospholipases, cyclooxygenase, and lipoxygenase enzyme families. While some studies have shown significant incorporation of 9,11 CLA into mammary and forestomach phospholipids (9,19), others have suggested that the chemoprotective effects of CLA in mammary gland and colon are due to incorporation of CLA into neutral lipids of nontumor-bearing tissue (4,5). Cook *et al.* (23) recently showed dietary CLA (0.5%; w/w) partially displaced arachidonate in rat fat pads; tissue CLA increased and arachidonate decreased significantly ($P < 0.05$) while linoleate decreased slightly. Numerous studies have shown dietary lipids (i.e., n-3 rich PUFA or linoleate-rich diets) result in decreased phospholipid arachidonate with concurrent reduction of arachidonate-derived eicosanoid synthesis and tumorigenesis. Therefore, CLA reduction of phospholipid arachidonate is likely to inhibit eicosanoid synthesis and affect tumorigenesis. The effects of dietary CLA on eicosanoid synthesis are not yet known.

Our studies are the first to show that diets containing incremental levels of CLA with constant amounts of linoleate in the form of corn oil (~2.8% by weight of all diets) are associated with accumulation of both CLA and oleic acid in hepatic neutral lipid fractions. Accumulation of fatty acids appears to occur with a concurrent reduction of linoleate. Previous studies using similar diet designs have demonstrated CLA deposition into liver tissue was not at the expense of altered ratios of other fatty acids (i.e., linoleate and oleate); however, these earlier studies did not report all major fatty acid constituents of neutral and phospholipid fractions but rather reported selected fatty acids including CLA, linoleate, γ-linolenate, and/or arachidonate (5,8). Three lines of evidence suggest that CLA may

TABLE 4
Effect of Experimental Diets on Fatty Acid Composition[a] of Mouse Liver

	Neutral lipids				Phospholipids			
	Diet group				Diet group			
Fatty acid	A	B	C	D	A	B	C	D
14:0	0.51 ± 0.06	0.55 ± 0.07	0.61 ± 0.04	0.61 ± 0.06	0.18 ± 0.12	0.25 ± 0.20	0.26 ± 0.13	0.19 ± 0.19
16:0	26.16 ± 2.57	25.52 ± 0.50	27.28 ± 1.29	27.46 ± 1.76	22.85 ± 0.81	23.60 ± 1.6	22.18 ± 2.10	22.08 ± 3.12
16:1;9	3.56 ± 0.35	3.19 ± 0.21	3.31 ± 0.12	3.81 ± 0.21	1.53 ± 0.15	1.30 ± 0.3	1.22 ± 0.49	1.33 ± 0.68
18:0	4.99 ± 0.89[b]	4.83 ± 0.26[b]	3.83 ± 0.49[c]	3.64 ± 0.58[c]	16.93 ± 1.43	16.63 ± 2.97	17.32 ± 3.90	15.99 ± 2.10
18:1; 9	42.85 ± 1.02[b]	44.28 ± 1.34[b]	44.50 ± 2.15[b]	47.63 ± 2.05[c]	22.03 ± 2.58	20.48 ± 3.03	20.09 ± 3.50	21.95 ± 5.19
18:1; 11	4.82 ± 0.22	4.56 ± 0.17	4.45 ± 0.79	4.96 ± 0.16	3.86 ± 0.26	3.44 ± 0.16	3.62 ± 0.29	3.26 ± 1.48
18:2; 9,12 (LA)	10.63 ± 0.58[b]	9.55 ± 1.00[b]	8.20 ± 0.98[c]	5.97 ± 0.79[d]	11.94 ± 0.65[b]	11.32 ± 0.96[b]	10.64 ± 0.99[b]	8.59 ± 1.48[c]
18:2 (9,11 CLA)	0.06 ± 0.03[b]	0.47 ± 0.15[c]	0.67 ± 0.07[d]	0.65 ± 0.09[d]	0.0[b]	0.13 ± 0.11[c]	0.28 ± 0.25[c,d]	0.38 ± 0.22[d]
18:2 (10,12 CLA)	0.0[b,e]	0.17 ± 0.04[c]	0.35 ± 0.02[d]	0.38 ± 0.05[d]	0.0	0.14 ± 0.14	0.20 ± 0.19	0.10 ± 0.14
20:1;11	0.40 ± 0.02	0.36 ± 0.04	0.45 ± 0.11	0.41 ± 0.03	0.18 ± 0.23	0.16 ± 0.14	0.30 ± 0.07	0.10 ± 0.14
20:4; 5,8,11,14	4.29 ± 1.78[b]	4.58 ± 0.67[c]	3.02 ± 0.58[d]	2.87 ± 0.71[d]	20.15 ± 3.40	22.08 ± 3.12	21.72 ± 4.44	19.14 ± 2.54

[a]Values are means ± SD expressed as percentage total fatty acid. n = 5–6 liver samples per diet group. Means with different superscripts ([b,c,d]) within the same row within neutral or phospholipid samples (Diets A–D) are significantly different ($P < 0.05$). [e]Not detected in measurable quantities and estimated to account for less than 0.09% total fatty acid. See Table 1 for abbreviations.

compete with linoleate for Δ6 desaturase, the rate-limiting step in the conversion of linoleate to form arachidonate. First, when available as a sole substrate, our studies demonstrate radiolabeled ^{14}C-CLA was desaturated to a similar extent as ^{14}C-linoleate by Δ6 desaturase. Second, dietary CLA (1.0 and 1.5%, Diets C and D) was associated with reduced arachidonate in mouse liver, suggesting less linoleate was converted to arachidonate. Finally, because oleate is a substrate for Δ6 desaturase, dietary CLA induction of oleate accumulation in total and neutral lipids suggests CLA was desaturated at the expense of oleate. Together these data suggest that dietary CLA modulation of Δ6 desaturase activity may reduce phospholipid-associated arachidonate, resulting in an overall decrease in arachidonate-derived eicosanoids.

In the present and previous studies from our laboratory (2), dietary CLA has been associated with reduced body weights in female SENCAR mice independent of food intake. These results are in contrast with findings in female rats where dietary CLA has displayed no significant effect on body weights (3,4,7,8,24). In addition to effects on body weights, results from our lab demonstrate that dietary CLA is associated with increased liver lipid accumulation. To our knowledge, effects of dietary CLA on lipid concentrations in rodent liver have not previously been reported; however, recent studies from Pariza and coworkers have suggested that CLA modulates fat and protein metabolism in mice, rats, and chickens (24). Furthermore, a hypolipidemic effect has been associated with diets containing 1.0% CLA compared to diets without CLA in rabbits (25). These data together suggest that dietary CLA has a potent effect on lipid transport and metabolism *in vivo.*

CLA has several physiological (i.e., reduced body weights and enhanced hepatic lipid content) and structural similarities to the fatty acid-like class of chemicals known as peroxisome proliferators. Recent findings from our lab have demonstrated that diets containing 1.0 and 1.5% CLA were associated with hepatic peroxisome proliferation (Belury, M.A., and Vanden Heuvel, J.P., submitted for publication). Peroxisome-specific enzymes include acyl-CoA oxidase, the rate-limiting enzyme in peroxisomal β-oxidation (26), liver fatty acid binding protein, a cytosolic lipid transport protein thought to be involved with shuttling fatty acids to and from the plasma membrane (27), and cytochrome p450IVA1, a lipid-metabolizing enzyme involved in ω-hydroxylation of fatty acids (28). Because each of these peroxisome-specific enzymes contribute to β-oxidation of fatty acids, peroxisomes are likely to be involved in maintaining cellular lipid homeostasis. Although the role of CLA in lipid metabolism is largely unknown, induction of peroxisome-specific fatty acid catabolic enzymes may shed some light on how CLA modulates fatty acid metabolism and carcinogenesis in extrahepatic tissues.

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Linear, Steroidal, and Triterpene Esters, and Steryl Glycosides from *Festuca argentina*

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ABSTRACT: Ester waxes and steryl glycosides of the grass *Festuca argentina* were studied. Saponification of the waxes from the petroleum ether extract led to *n*-hexacosanol as the major single linear alcohol, along with pentacyclic triterpenols, such as β-amyrin, germanicol, isobaurenol, lupeol, hopenol-a and hopeol, and low amounts of sterols, such as cholesterol, campesterol, stigmasterol, sitosterol and dihydrositosterol, identified by gas chromatography/mass spectrometry (GC/MS). Fatty acids were identified as methyl esters as $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:2}$, and $C_{20:0}$. The occurrence of a wide chainlength range of fatty acids and a single linear alcohol closely matched for other reports on the tribe Festuceae. On the contrary, pentacyclic triterpenols with a variety of skeletons, especially isobauerenol, are not usual as esters of fatty acids in the Gramineae. Low amounts of steryl glycosides were also obtained from the methylene chloride percolate of the methanol extract. Upon acetylation followed by hydrolysis, aglycones were identified by capillary gas–liquid chromatography (GLC) and GC/MS. As Δ^7-cholesterol, campesterol, stigmasterol, sitosterol, dihydrositosterol, and the sugars as glucose, xylose, and arabinose by GLC of the respective alditol acetates. This is the first report on the linear, steryl, and triterpenyl esters of *F. argentina*. It is noteworthy that Δ^7-steryl glycosides are rare, and steryl monoarabinosides have not been previously reported on the family Gramineae.

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Festuca argentina (Speg.) Par. (Gramineae; common name: "coirón negro," "coirón duro") is a perennial xerophyte Patagonian grass popularly known as toxic to sheep (1) that grows in the pre-Andinian region, and eastward around the Gulf of San Jorge (2) in the Patagonian coast of Argentina.

We have previously reported on free sterols and triterpenes from this species (3). The Δ^5-, Δ^7-, and dihydrosterols were identified as well as pentacyclic triterpenones with a variety of skeletons, such as oleanene/ursene (Δ^{12}-oleanen-3-one or β-amyrenone, Δ^{18}-oleanen-3-one = germanicone), lupene (lupen-3-one), hopene ($\Delta^{22(29)}$-hopen-3-one) and multiflorene (Δ^8-multiflorenone = isomultiflorenone) and a cyclopropane tetracyclic triterpenone (cycloartanone), the two latter being rare in the Gramineae. However, the expected 4-methylsterols, 3-methoxytriterpenes and arborene and/or fernene triterpenoids were not detected in this species, although widespread in the Gramineae (4).

In continuation of our work on members of the tribe Festuceae, we want to report in this paper the composition of the ester waxes and steryl glycosides of *F. argentina* that, in contrast to other grasses, showed to be rich not only in a linear alcohol but also in both bound sterols and triterpenols.

Wax esters from other members of the Gramineae usually accounted for esters of fatty acids–*n*-alcohols, e.g., *Bromus inermis*, *F. ovina* (5), *Secale cereale* (6), three wheat species (7–9), and *Agropyron desertorum* and *A. intermedium* (10), due to interesterification of a wide range of acids with a much smaller chainlength range *n*-alcohols. Combined alcohols from waxes of festucoid grasses (5,11) generally contain one major component, C_{26} or C_{28}, with the same chainlength as the major free alcohol of the wax. However, in *Agropyron smithii* (tribe Triticeae) a wide chainlength range of both acids and alcohols from the ester waxes were obtained without any prevalent long-chain major component (12), the alcohol portion also containing unusual appreciable percentage of α- and β-amyrins (40%). A wide range of chainlength linear alcohols and triterpenol esters was also typical of species of the subfamily Eragrostoideae (13).

Detailed information on the identities of naturally-occurring phytosterols as glycosides is far from complete. So far, up to now these compounds have not been described as characteristic of any family, and they are scarcely distributed, except for sitosterol 3-*O*-glucoside, in agreement with earlier biosynthetic studies in *Triticum aestivum*, showing that UDP-glucose is the most active glycosyl donor (14). We have detected in *F. argentina* several steryl glycosides composed of a variety of steryl and sugar moieties. The occurrence of these compounds may be related to a sterol storage function, instead of simple waste products as considered by some plant physiologists especially interested in the role of free sterols (14).

MATERIALS AND METHODS

Plant material. Plants of *F. argentina* (Speg.) Par. (family: Gramineae) were collected by Prof. Ing. Angel Soriano in Río

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Abbreviations: GC/MS, gas chromatography/mass spectrometry; GLC, gas–liquid chromatography; MPLC, medium-pressure liquid chromatography; MS, mass spectra; MW, molecular weight; PE, petroleum ether; R_f, retention front; R_t, retention time; TLC, thin-layer chromatography.

Mayo (EE-INTA Río Mayo; Patagonia), Province of Chubut, Argentina, and identified by Dr. Elisa Nicora (Instituto de Botánica Darwinion, San Isidro, Argentina); voucher specimen were deposited in the Instituto de Botánica Darwinion (Argentina) SI 28004. This is a monocotyledonous matted grass with blades, roots, and spikelets with minute structures which are important markers of taxa. Spikelets of grass species within a genus differ in the rachilla joints, glumes, lemmas, or paleas. In addition it has a distinct festucoid-type embryo. The plant material used in this study was harvested in February (summer season in southern hemisphere) in the so-called fruit stage/period; it was composed of leaves (blades) and adventitious fibrous roots, and devoid of spikelets.

Materials and chromatographic conditions. Analytical thin-layer chromatography (TLC) plates (silica gel G, F-254, 250 μm layer), silica gel G and silica gel H were purchased from Merck (Darmstadt, Germany); polyamide for chromatography was purchased from Woelm (Eschwede, Germany). Preparative TLC (20 cm × 20 cm; 1000 μm layer) were prepared in our laboratory using a suspension of silica gel G (30 g) in distilled water (60 mL). Freshly prepared plates were kept at room temperature for 24 h and further activated at 110–120°C for 1 h prior to use. Spots were visualized under ultraviolet (UV) light at 254 nm and/or by spraying the plates with H_2SO_4/AcOH (1:1) and heating at 110–120°C for 2 min. Medium-pressure liquid chromatography (MPLC) was carried out on silica gel H (Merck) columns with an impulse solvent bomb Prominent-Electronic 1001 SCJ (Heidelberg, Germany) with a pulse damper. Flash dry chromatography was performed under vacuum through a fritted Pyrex glass funnel membrane (Thomas Scientific, Swedesboro, NJ). Organic solvents were obtained from Sintorgan (Buenos Aires, Argentina) followed by distillation, except for petroleum ether (PE) and *n*-hexane which were freed from olefines in the usual way prior to distillation. All solvent mixtures are expressed in volume (vol/vol).

Extraction of the plant material. As soon as it was collected, fresh material was air-dried in the laboratory in an air-circulating oven at 25°C to remove soil adhered to roots and blades; then it was ground and immediately extracted in a Soxhlet with PE (60–80°C) followed by MeOH. Flash dry chromatography of PE extract (1.6 % relative to dry weight) using gradients of *n*-hexane/EtOAc as previously reported (3) followed by TLC analysis led to six main fractions, termed 1-6. TLC (*n*-hexane/EtOAc, 98:2) of fraction 2 gave a main pink spot at R_f 0.60 typical of waxes, a brown spot at R_f 0.53 due to aldehydes, and a light-orange spot at R_f 0.38 due to terpenoids. Separation of these components (subfractions 2a, 2b, and 2c) was afforded by repeated preparative TLC (*n*-hexane/EtOAc, 97:3). Bands were eluted with chloroform and monitored by analytical TLC, and the waxes were characterized by infrared spectra (KBr) using a Perkin-Elmer 710-B spectrophotometer (Palo Alto, CA). The MeOH extract was further percolated on polyamide with CH_2Cl_2, H_2O, and finally MeOH, rendering 1.0, 2.8, and 2.3% relative to dry plant, respectively. The CH_2Cl_2 percolate was worked up as described before (15) yielding a subfraction 2 that was purified by MPLC (EtOAc). A Liebermann-Burchard positive (16) fraction that gave a diffuse pink spot of R_f 0.65 by TLC (EtOAc/MeOH, 95:5) contained steryl glycosides (3 mg; 0.005% relative to dry plant) which were acetylated (Ac_2O/anhydrous pyridine) to overcome their insolubility in organic solvents (R_f 0.80 and 0.85, high-performance thin-layer chromatography: EtOAc/MeOH, 95:5), and further hydrolyzed.

Saponification of waxes. Saponification was performed by stirring the waxes (0.13 g) under reflux with 20% KOH in MeOH for 4 h. The reaction was monitored by TLC. The mixture was then evaporated to dryness. Water was added to the residue and the aqueous layer was further extracted with $CHCl_3$. The organic layers were combined, dried over anhydrous Na_2SO_4 and the chloroform was evaporated under vacuum to give the alcohols. The remaining aqueous layer was acidified and the acidic compounds were extracted with $CHCl_3$. This chloroform layer was dried over anhydrous Na_2SO_4 and then evaporated to dryness to give the organic acids. These organic acids were methylated (CH_2N_2/Et_2O) for 14 h at room temperature.

Hydrolysis of the steryl glycosides. The acetylated glycosides were hydrolyzed with 6% HCl in MeOH. The reaction mixture was heated until dissolution of the glycosides and drops of water were added until there was slight turbidity. Then it was kept at 75°C in a sealed tube for 2 h. After neutralization with $NaHCO_3$ and evaporation of the solvent, the residue was partitioned with CH_2Cl_2/H_2O (1:1). The CH_2Cl_2 layer was dried with anhydrous Na_2SO_4 and evaporated at 40°C, yielding sterols. The aqueous layer containing the sugars was desalted with Amberlite MB-3/thymolphthalein (Sigma, St. Louis, MO) to prepare the alditol acetates ($NaBH_4$, room temperature, 12 h; Ac_2O/pyridine, 1:0.8).

Gas–liquid chromatography (GLC) and gas chromatography/mass spectrometry (GC/MS) analysis of the compounds. GLC was performed on a Hewlett-Packard 5840 A chromatograph (Palo Alto, CA) with flame-ionization detection (FID) and nitrogen as gas carrier. Capillary GC/MS were performed at 70 eV on a Hewlett-Packard 5890A chromatograph coupled with a selective mass detector 5970B. Mass spectra (MS) data were processed by a Lab Base GC-MS data system (40 to 800 mass scanning). The respective alcohol mixtures from the hydrolysis of the waxes and of the glycosylsterols were chromatographed on a bonded-phase fused silica capillary column (DB-1, 30 m length × 0.245 mm id, 0.25 μm film thickness; J&W Scientific, Folsom, CA). Oven temperature was set at 180°C for 1 min with a 5°C/min temperature ramp up to a final temperature of 300°C for the alcohols from the ester waxes, and 200–300°C, rate 10°C/min for the aglycones from the glycosylsterols. The identities of *n*-hexacosanol, β-amyrin, germanicol, lupeol and hopeol, as well as the sterols were confirmed by capillary GLC with standards. In addition, the mixtures were examined by GC/MS on a 25 m × 0.25 mm, 0.33 μm film thickness Ultra-1 capillary column, temperature programmed from 240–300°C at 3°C/min for wax alcohols, and 200–300°C, rate 15°C/min for glycosides aglycones.

Fatty acids from the wax hydrolysis were separated as methyl esters on a 1.8 m × 2 mm 8% NPGS packed column on Chromosorb W-AW-DMCS, 60-80 mesh, at 190°C, using authentic fatty acid methyl ester standards. Identification was confirmed by GC/MS spectrometry on a 1.2 m length × 2 mm i.d. SP-2340 column (Supelco, Bellefonte, PA) programmed from 60–270°C at 8°C/min. Sugars from steryl glycosides were analyzed by capillary GLC of their alditol acetates against synthetic standards (SP 2330, 30 m length × 0.25 mm id, 0.20 μm film thickness; Supelco).

RESULTS AND DISCUSSION

Ester waxes. Repeated chromatographic purification techniques applied to the PE extract of *F. argentina* yielded a subfraction with an infrared spectrum typical of high molecular weight (MW) ester waxes [2895 ($\nu_{C\text{-}H}$), 1742 ($\nu_{C=O}$), 1475 ($\delta_{C\text{-}H}$), 1180 ($\nu_{C\text{-}O}$) and 740 cm^{-1} ($\delta_{C\text{-}H}$)]. Upon hydrolysis, a homologous series of saturated fatty acids with even carbon atom from C_{12} to C_{20} was obtained, which showed typical fragmentation pattern (17) by GC/MS analysis of their respective methyl esters with *m/z* 74 as base peak, and M^+ at *m/z* 214, 242, 270, 298, and 326, respectively. In addition, M^+ 294 was shown to be a fatty acid of C_{18} containing two unsaturations ($C_{18:2}$) by its mass fragmentation pattern. The acid composition of the ester waxes from *F. argentina* is shown in Table 1, with hexacosanoic acid being the major component among the seven identified fatty acids.

By contrast, the wax alcohols (Table 1; Fig. 1) showed a single major linear alcohol, *n*-hexacosanol ($C_{26:0}$), which accounted for 61% of the total alcohol wax composition, along with triterpene alcohols (31.2%) and low amounts of sterols (7.6%) (Fig. 1A). The low levels of triterpenols and sterols were detected by GC/MS in the 15 to 24 min region of the amplified chromatogram (Fig. 1B). The MS of the respective sterols showed M^+ 386, 400, 412 and 414, and the fragmentation pattern typical of Δ^5-sterols (Table 2) (18), corresponding to cholesterol, campesterol, stigmasterol, sitosterol, along with another sterol of R_t and M^+ higher than that of sitosterol, and an intense ion *m/z* 233 in agreement with dihydrositosterol (19). The GC/MS of the pentacyclic triterpenoid alcohols were in the range of retention time (R_t) 20.34 to 22.99 min. The compound of R_t 20.34 min, M^+ 426, and *m/z* 218 as base peak showed a fragmentation pattern (Table 2) of a Δ^{12}-ursene/Δ^{12}-oleanene triterpenol (20), confirmed as β-amyrin by capillary GLC with authentic samples, due to the fact that both skeletons are undistinguishable by MS. The compound of R_t 20.52 min (Table 2) presented a M^+ 426 followed by loss of 15 amu, and an intense ion at *m/z* 177 (98.5%) typical of a Δ^{18}-oleanene or Δ^{18}-friedelene (20); the low abundance (14.7%) of ion *m/z* 203, usually very high in Δ^{18}-friedelene (20) provided evidence for a Δ^{18}-oleanene, further confirmed by GLC comparison with a germanicol sample. The compound of R_t 20.93 min, M^+ 426, *m/z* 247 as base peak and other ions (Table 2) was characteristic (21) of a D:C-friedo-oleanane/D:C-friedo-ursane skeleton with one unsaturation either at Δ^7-(multiflorenol/bauerenol) or at Δ^8-(isomultiflorenol/isobauerenol), prevailing the bauerene series (20) by the intense *m/z* 247 and the low abundant *m/z* 205. Comparison of relative abundances was required for the Δ^7/Δ^8 location of the double bond, e.g., base peak, M^+ and M^+-Me of isobauerenol (22) and β-bauerenol (23), thus leading to isobauerenol (bauer-8-en-3-β-ol) only once reported in Gramineae (24). Compounds with R_t 21.18, 22.76, and 22.99 min showed similar MS (Table 2) concerning the molecular ion (M^+ 426) and the highly abundant *m/z* 207, arising from hydroxyl-containing rings A/B of saturated triterpenols without directing groups. The fact that *m/z* 189 is more intense than *m/z* 191, along with the low intensity or absence of M^+–

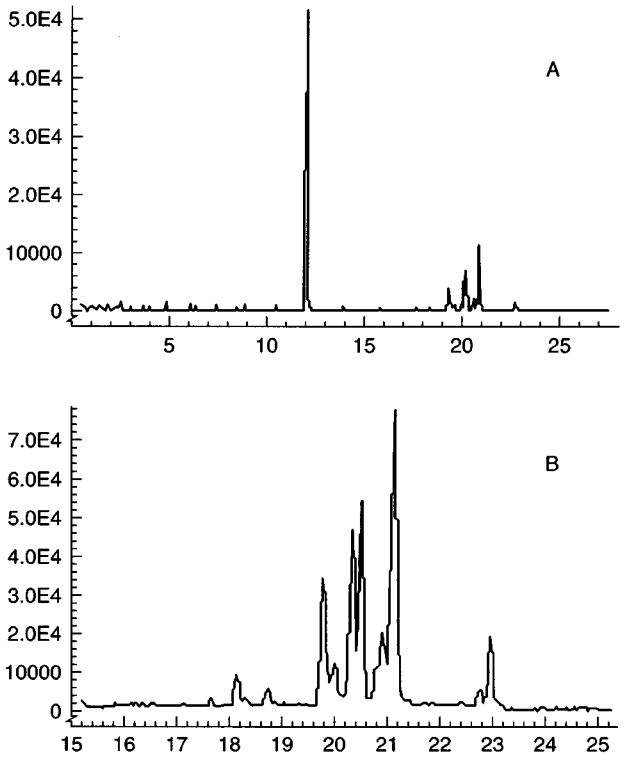

FIG. 1. Gas chromatography/mass spectrometry of the alcohols from ester wax hydrolysis. (A) general chromatogram; (B) chromatogram of the amplified 15 to 24 min region.

TABLE 1
Composition of Acids and *n*-Alcohols from *Festuca argentina* Waxes

	12:0	14:0	16:0	18:0	18:2	20:0	26:0
Esterified acids[a]	1.9	4.0	38.8	25.7	19.8	5.7	—
Esterified alcohols[b]	—	—	—	—	—	—	61.0

[a]Fatty acids analyzed as methyl esters by gas–liquid chromatography (GLC) on 8% NPGS and comparison with standards. Identification confirmed by gas chromatography/mass spectrometry (GC/MS) on a SP-2340 column. For details, see the Materials and Methods section.

[b]Alcohols were analyzed by capillary GLC on DB-1. Identification of *n*-hexacosanol is based on cochromatography with a known standard and GC/MS as indicated in Table 2.

TABLE 2
Alcohols from the Saponification of *Festuca argentina* Waxes

Alcohol[a]	R_t (min)	Characteristic mass peaks (*m/z*) (% relative abundance)
n-Hexacosanol	12.52	364 ($M^+ - H_2O$, 5.2), 336 (364 – $CH_2 = CH_2$, 2.5), 308 (0.5), 280 (0.8), 266 (0.7), 252 (0.9), 238 (1.0), 224 (1.2), 210 (1.5), 195 (2.3), 181 (3.3), 167 (4.5), 153 (6.6), 139 (10.6), 125 (19.7), 111 (37.2), 97 (72.9), 83 (83.7), 73 (1.5), 71 (56.9), 69 (69.0), 57 (100.0), 55 (62.2), 43 (76.9), 41 (21.9).
Cholesterol	17.59	387 ($M^+ + 1$, 29.2), 386 (M^+, 100.0), 371 (M^+ – Me, 31.2), 368 ($M^+ - H_2O$, 39.7), 353 ($M^+ - H_2O$ – Me, 32.5), 301 (M^+ – 85, 49.1), 275 (M^+ – 111, 37.5), 273 (M^+ – side chain, 21.0), 247 (M^+ – 139, 7.4), 231 (M^+ – side chain – 42, 17.9).
Campesterol	18.10	401 ($M^+ + 1$, 29.6), 400 (M^+, 100.0), 385 (M^+ – Me, 29.3), 382 ($M^+ - H_2O$, 39.9), 367 ($M^+ - H_2O$ – Me, 28.5), 315 (M^+ – 85, 43.2), 289 (M^+ – 111, 27.6), 273 (M^+ – side chain, 18.9), 261 (M^+ – 139, 5.5), 231 (M^+ – side chain – 42, 16.8).
Stigmasterol	18.68	413 ($M^+ + 1$, 23.0), 412 (M^+, 68.9), 397 (M^+ – Me, 6.1), 394 ($M^+ - H_2O$, 5.0), 379 ($M^+ - H_2O$ – Me, 7.3), 369 (M^+ – 43, 8.9), 351 (369 – H_2O, 15.8), 300 (M^+ – 112, 20.2), 271 (M^+ – side chain – 2H, 19.6).
Sitosterol	19.79	415 ($M^+ + 1$, 19.7), 414 (M^+, 63.1), 399 (M^+ – Me, 16.9), 396 ($M^+ - H_2O$, 24.7), 381 ($M^+ - H_2O$ – Me, 17.5), 329 (M^+ – 85, 24.4), 303 (M^+ – 111, 12.5), 275 (M^+ – 139, 3.3), 273 (M^+ – side chain, 10.9).
Dihydrositosterol	19.99	417 ($M^+ + 1$, 30.8), 416 (M^+, 100.0), 401 (M^+ – Me, 38.8), 398 ($M^+ - H_2O$, 2.7), 383 ($M^+ - H_2O$ – Me, 14.9), 234 (M^+ – side chain – 41, 48.2), 233 (M^+ – side chain – 41 – H, 75.2), 215 (57.8).
β-Amyrin	20.34	427 ($M^+ + 1$, 0.6), 426 (M^+, 2.7), 411 (M^+ – Me, 0.5), 393 (M^+ – Me – H_2O, 0.9), 219 (18.7), 218 (RDA, rings D and E, 100.0), 205 (rings D and E or rings A and B, 0.5), 203 (218 – Me, 39.3), 189 (207 – H_2O and/or rings D and E – 29, 4.5).
Germanicol	20.52	427 ($M^+ + 1$, 8.5), 426 (M^+, 31.7), 411 (M^+ – Me of C – 17, 26.0), 393 (M^+ – Me – H_2O, 1.9), 218 (rings D and E + C – 11 + C – 12,), 207 (rings A and B, 19.7), 206 (15.3), 205 (rings D and E + C – 12, 37.6), 204 (rings D and E + C – 12, 100.0), 203 (218 – Me, 14.7), 191 (18.1), 190 (205 – Me, 24.9), 189 (93.8), 178 (21.4), 177 (RDA in ring D + C – ring, 98.5).
Isobauerenol	20.93	427 ($M^+ + 1$, 7.8), 426 (M^+, 21.9), 411 (M^+ – Me, 14.1), 393 (M^+ – Me – H_2O, 5.1), 259 (rings A + B + C + Me – 26, 15.5), 247 (RDA ring C and rupt. C – 15 – C – 16, 100.0), 229 (57.3), 205 (rings D and E, 8.8).
Lupeol	21.18	427 ($M^+ + 1$, 12.0), 426 (M^+, 38.0), 411 (M^+ – Me, 13.4), 408 ($M^+ - H_2O$, 1.7), 393 (M^+ – Me – H_2O, 3.2), 383 (M^+ – isopropyl, 3.8), 218 (rings D and E, 89.3), 207 (rings A and B, 59.3), 204 (28.7), 203 (37.4), 191 (26.8), 190 (7.8), 189 (60.8),135 (64.1), 121 (65.3), 109 (70.9), 108 (47.9), 107 (78.8), 105 (46.5), b.p. 43.
Hopenol-a	22.76	427 ($M^+ + 1$, 18.0), 426 (M^+, 47.8), 207 (rings A and B, 85.7), 204 (24.2), 191 (35.4), 190 (43.5), 189 (207 – H_2O and/or rings D and E, 100.0).
Hopeol	22.99	427 ($M^+ + 1$, 11.9), 426 (M^+, 36.6), 218 (16.9), 217 (rings D and E + C – 11 + C – 12, 7.2), 208 (RDA, rings A and B, 20.6), 207 (79.6), 205 (22.8), 204 (24.3), 203 (22.9), 191 (32.5), 190 (33.4), 189 (RDA, rings D and E – H, 89.1), 135 (60.0), 121 (70.3), 109 (86.6), 107 (76.5), 105 (33.4), b.p. 43.

[a]Identification confirmed on a 25-m capillary column Ultra-1 by GC/MS (EI-70 eV). For details, see the Materials and Methods section. See Table 1 for abbreviation.

43, is indicative of an isopropenyl group in the molecule, thus supporting the occurrence of either lupane or hopane skeletons, which usually lack a characteristic fragmentation pattern. Consequently, the identity of compounds with R_t 21.18 and 22.99 min was confirmed by capillary GLC comparison with authentic samples of lupeol and hopeol (hopenol-b = hop-22(29)-en-3-β-ol), respectively. The MS of the minor compound of R_t 22.76 min showed only two fragments in the high-mass region suggesting a difference in the double-bond location of the isopropenyl group relative to the two latter compounds, thus leading to hopenol-a.

Steryl glycosides. Successive MPLC purification steps of the MeOH extract of *F. argentina* gave rise to a Liebermann-Burchard positive fraction (16) composed of steryl glycosides, which accounted for 0.005% relative to dry weight. Its chromatographic behavior (TLC, R_f region) was in agreement with nonacylated steryl monoglycosides. No changes were observed upon *O*-methylation (ethereal CH_2N_2), indicating the absence of uronic acids (25). After acid hydrolysis of the acetate derivatives, both sterols (aglycones) and sugars (as alditol acetates) were identified by capillary GLC and capillary GC/MS analysis. Glucitol (16.4%), xylitol (28.9%), and

arabinitol (54.7%) were characterized and further confirmed by capillary GLC comparison with synthetic standards. Accordingly, 3-*O*-monoglycosides of cholesterol (11.3%), Δ^7-cholesterol (14.2%), campesterol (13.9%), stigmasterol (14.5%), Δ^7-sitosterol (15.3%), sitosterol, and 5,6-dihydrositosterol (30.8%) occur in *F. argentina*, some of them as 3-*O*-monoglucosides, 3-*O*-monoxylosides, and 3-*O*-monoarabinosides. Although sitosterol 3-*O*-glucopyranoside is widespread in nature (26–28), only few other Δ^5-steryl 3-*O*-monoglycosides have been reported, such as stigmasterol 3-*O*-glucopyranoside (29), sitosterol 3-*O*-β-D-xylopyranoside (30), sitosterol 3-*O*-α-D-riburonofuranoside (25), sitosterol 3-*O*-α-D-xyluronofuranoside (31), and sitosterol 3-*O*-β-D-glucuronopyranoside (32). To the best of our knowledge, the isolation and identification of monoglycosides of dihydrosterols and Δ^7-sterols from natural sources is rare, such as 3-*O*-β-D-glucopyranoside and 3-*O*-β-D-galactopyranoside of (24*R*)-stigmast-7,22(*E*)-dien-3-α-ol (epichondrillasterol) (33). Moreover, D-glucose is the usual sugar moiety (34) of monoglycosides of a variety of Δ^5-phytosterols, steryl 3-*O*-monoarabinosides being so rare in nature that stigmasterol α-L-arabinopyranoside has only been isolated once, from *Wallenia yunquensis* (35).

In summary, free (3), esterified and glycosylated 4-demethylsterols were found in *F. argentina*, whereas pentacyclic triterpenols merely occurred as esters of high-MW fatty acids. These findings closely resemble earlier reports on *Sorghum vulgare* (36). Thus, our results show that Δ^5-sterols and dihydrositosterol are found in free (3), esteryl and glycosyl forms, while Δ^7-sterols are present only free (3) and/or as glycosyl derivatives. Nevertheless, the relative percentage of the sterols in free and bound forms are distinctly variable, e.g., free cholesterol (1.3%) (3), glycosyl cholesterol (11.3%), and only traces of esterified cholesterol. Conversely, the proportion of free sitosterol and dihydrositosterol appears to be higher (56.3%) than the glycosylated (30.8%) and esterified (6.46%) forms.

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[3-^{13}C] γ-Linolenic Acid: A New Probe for ^{13}C Nuclear Magnetic Resonance Studies of Arachidonic Acid Synthesis in the Suckling Rat

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ABSTRACT: Our objective was to develop a suitable probe to study metabolism of polyunsaturated fatty acids by ^{13}C nuclear magnetic resonance (NMR) in the suckling rat pup. [3-^{13}C] γ-Linolenic acid was chemically synthesized, and a 20 mg (Experiment 1) or 5 mg (Experiment 2) dose was injected into the stomachs of 6–10-day-old suckling rat pups that were then killed over a 192 h (8 d) time course. ^{13}C NMR showed that ^{13}C in γ-linolenate peaked in liver total lipids by 12-h post-dosing and that [5-^{13}C]-arachidonic acid peaked in both brain and liver total lipids 48–96 h post-dosing. ^{13}C enrichment in brain γ-linolenic acid was not detected by NMR, but gas chromatography–combustion–isotope ratio mass spectrometry showed that its mass enrichment in brain phospholipids at 48–96 h post-dosing was 1–2% of that in brain arachidonic acid. ^{13}C was present in liver and brain cholesterol and in perchloric acid-extractable water-soluble metabolites in the brain, liver and carcass. We conclude that low but measurable amounts of exogenous γ-linolenic acid do access the suckling rat brain *in vivo*. The slow time course of [5-^{13}C] arachidonic acid appearance in the brain suggests most of it was probably transported there after synthesis elsewhere, probably in the liver. Some carbon from γ-linolenic acid is also incorporated into lipid products other than n-6 long-chain polyunsaturated fatty acids.

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γ-Linolenic acid (18:3n-6; GLA) is formed by desaturation at the C6–C7 bond of linoleic acid (18:2n-6) and is an intermediate fatty acid in the desaturation/chain-elongation pathway between linoleic and arachidonic acid (20:4n-6; AA) (1). In mammals, GLA is usually present in tissue lipids in low amounts, mainly because it appears to be readily converted to dihomo-γ-linolenate (20:3n-6) before subsequent, slower desaturation to AA (2). GLA is not commonly found in the diet, but it is present in human breast milk. Concentrated edible sources of GLA are mostly limited to the oil in the seeds of the evening primrose, black currant, or borage plants (3).

AA is the main n-6 polyunsaturated fatty acid (PUFA) in the brain, but the origin of brain AA has not been established, i.e., the relative contributions of diet, synthesis outside the brain or synthesis within the brain are not known. Establishing the dependence of the brain on pre-formed AA would help determine the need to provide AA or closer precursors than linoleic acid in the diet. AA is present in small amounts in adipose tissue and milk, but it can also be synthesized in organs such as the liver, gut, or in the brain itself. Brain levels of GLA are usually <0.2% of total fatty acids, but whether this is due to low access of GLA to the developing brain or to rapid conversion to n-6 long-chain (LC) PUFA is unknown at present. Dietary GLA is known to increase the level of AA in liver and other organs of experimental animals (4,5), but whether it affects the amount of AA destined for the developing mammalian brain is unknown. More radiolabeled GLA than linoleic acid was incorporated into brain AA in the suckling rat, but the time course of its conversion to AA and the relative importance of the liver were not reported (6).

Our objective was to develop a probe of neonatal AA synthesis that could be detected by noninvasive nuclear magnetic resonance (NMR) methodology and was safe for potential use in humans. ^{13}C is a stable isotope safe for human use and by locating the ^{13}C-enriched carbon at C-3 of GLA, the ^{13}C enrichment is detectable by *in vivo* ^{13}C-NMR spectroscopy in living rats (7). In the present study, we have used high-resolution ^{13}C-NMR to detect the original [3-^{13}C]-GLA vs. its conversion to [5-^{13}C]-AA. Gas chromatography (GC)–combustion–isotope ratio mass spectrometry (GC–C–IRMS) provided precise enrichment values for the ^{13}C-labeled fatty acids. These analytical methods offer complementary data on carbon-by-carbon ^{13}C enrichment and precise quantitation of ^{13}C enrichment, respectively (8–10).

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Abbreviations: AA, arachidonic acid; APE, atom % excess; GC, gas chromatography; GLA, γ-linolenic acid; IRMS, isotope ratio mass spectrometry; LC, long chain; NMR, nuclear magnetic resonance; PUFA, polyunsaturated fatty acids.

METHODS

Synthesis of [3-^{13}C] γ-linolenate. Ethyl [3-^{13}C]-all *cis*-6,9,12-GLA was prepared using classical methodology developed for chemical synthesis of labeled PUFA (11,12). The all-*cis*-3,6,9-pentadecatrienol, easily obtained by catalytic hydrogenation of the C_{15}-triynol precursor (13), was converted *via* the corresponding mesylate to the labeled nitrile (14). Acidic methanolysis of the crude nitrile under carefully controlled reaction conditions provided the methyl all-*cis*-4,7,10-hexadecatrienoate (15) which was reduced to an alcohol. Chain elongation of the resulting C_{16}-alcohol, using malonic ester synthesis (16), provided GLA. After distillation and chromatographic purification, esterification under standard conditions gave the desired ethyl [3-^{13}C]-GLA. GC analysis indicated there was 88% all-*cis* GLA, 2.4% heptadecanoate, 4.0% linoleate, and 3.7% *trans*-GLA. Labeling at carbon-3 was calculated to be in excess of 99% as established by spectroscopic methods and microanalysis of ^{12}C (found 73.93% vs. 74.22% in theory), ^{13}C (found 4.23% vs. 4.22% in theory), and hydrogen (found 11.20% vs. 11.15% in theory). ^{13}C NMR of the [3-^{13}C]-GLA in chloroform solution showed one major peak at 24.7 ppm corresponding to the ^{13}C-enriched carbon atom, and some very low peaks corresponding to expected peaks for unenriched carbons of GLA plus some others that were unidentified (Fig. 1).

Tracer protocol and tissue collection. All procedures involving animals followed protocols approved by the University of Toronto Animal Care Committee. Pregnant Sprague-Dawley rats (Charles River, St. Constant, QC, Canada) were housed singly and had *ad libitum* access to tap water and rodent chow (Ralston-Purina, St. Louis, MO). Two experiments were performed. In the first experiment, 6-day-old suckling rats were dosed directly into the stomach with 20 mg of [3-^{13}C] GLA. The rat pups were killed in groups of 3–4 at the following times after dosing: 1, 2, 4, 6, 8, 10, 12, 24, 48, 96, or 192 h (8 d). Controls were also killed at matched ages (6, 7, 8, 10, or 14 d old). Following euthanasia and decapitation, the brains and livers were excised, weighed, and immediately immersed in liquid N_2 and then stored on dry ice for analysis. Prior to lipid extraction, the organs were pulverized to a powder under liquid N_2 using a mortar and pestle. In Experiment 1, brains and livers were pooled separately at each time point (n = 3–4/time point) to obtain the highest possible signal/noise ratio in NMR spectra. In Experiment 2, 5 mg [3-^{13}C]-GLA was dosed into 10-day-old suckling rat pups, and the time points studied were 48 and 96 h post-dosing with 2–4 separate measurements/time point.

Extraction and analysis of water- and lipid-soluble metabolites. For lipid extraction, the powdered organs were transferred to vials containing 20 volumes/tissue volume of chloroform/methanol (2:1), and the total lipid extracts were recovered by standard procedures (17). Water-soluble metabolites were extracted from organ samples collected in the first 10 h after dosing with [3-^{13}C] GLA while lipid-soluble metabolites were extracted from samples collected 12–192 h post-dosing.

^{13}C enrichment in an aliquot of the dried brain and liver lipid extracts was determined by continuous flow combustion (C)–IRMS [Metabolic Solutions, Merrimack, NH) using an automated elemental analyzer (model ANCA and 20-20 IRMS; Europa Scientific, Crewe, Cheshire, United Kingdom)]. A second lipid aliquot was applied to thin-layer chromatography plates and developed in a neutral lipid solvent system for separation of total phospholipids from free cholesterol. ^{13}C enrichment in the separated total phospholipids and cholesterol of brain and liver was also analyzed by IRMS (Metabolic Solutions).

A third aliquot was taken from the total lipid extracts at the time of maximal ^{13}C enrichment for measurement of ^{13}C levels in individual fatty acids in liver and brain. Fatty acid methyl esters were prepared, and carbon isotope ratios were

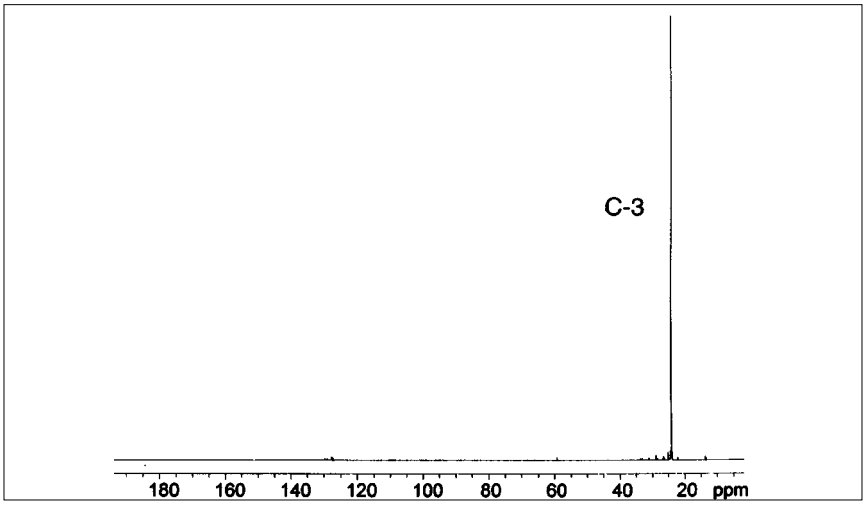

FIG. 1. ^{13}C nuclear magnetic resonance spectrum of [3-^{13}C]-γ-linolenic acid showing the ^{13}C-enrichment at 24.7 ppm which is assigned to C-3 of γ-linolenic acid.

determined by GC–C–IRMS as previously described in detail (9). Briefly, a GC (Varian Associates, Palo Alto, CA) operated in splitless mode was interfaced to an IRMS (Finnigan MAT 252; Bremen, Germany) *via* a combustion furnace/water trap system. The GC capillary column used was 0.25 mm × 60 m (DBWAX; J&W Scientific, Folsom, CA), and the oven temperature was programmed to ramp from 60 to 255°C with three plateaus. Isotope ratios were calibrated against a standard gas which was periodically calibrated against USGS 24 (National Bureau of Standards, Washington, D.C.). The standard gas was admitted when no analyte was entering the furnace and was integrated along with analyte peaks. The remainder of each lipid extract was dissolved in 500 µL deuterated chloroform (MSD Isotopes, Montreal, QC, Canada) and transferred to a 5-mm NMR tube. ^{13}C NMR spectra were obtained at 75 MHz (Bruker AM 300; Karlsruhe, Germany) as previously described (8).

To obtain water-soluble metabolites, organ samples were pulverized under liquid N_2 and homogenized in 6% perchloric acid. After centrifugation, the supernatants were neutralized, salts were removed on an ion exchange column, and the remaining material was freeze-dried. A sample of the dry, powdered perchloric acid extracts was assayed for ^{13}C content by combustion IRMS (Metabolic Solutions).

Data presentation. In the first experiment, organ weights, organ lipid content, and IRMS data are expressed as single values from 3–4 pooled samples because of the need to maximize the quality of ^{13}C NMR spectra by having the highest possible solute concentration. Isotope ratios were converted to atom % ^{13}C, and atom % excess (APE) was calculated by subtracting the atom % of the controls. In the second experiment, the data are given as mean ± SD for 2–4 samples/data point.

RESULTS

Experiment 1: ^{13}C NMR spectra of liver and brain lipids. ^{13}C NMR spectra of liver and brain total lipid extracts from rat pups dosed with ^{13}C-GLA at 6 d old had two particular regions of interest: the resonance for C-3 of GLA (24.7 ppm), and the resonance for C-5 of AA (128.9 ppm) (Figs. 2–4). The assignment of the peak at 24.7 ppm to C-3 of GLA was based on the ^{13}C-NMR spectrum of the pure tracer before dosing (Fig. 1). The assignment of C-5 of AA at 128.9 ppm is based on the metabolism of GLA in which C-3 becomes the C-5 olefinic carbon of AA; unequivocal assignments of the carbons of AA have not been reported to our knowledge. Control spectra of liver or brain total lipids had no detectable signal at C-3 of GLA. The C-3 signal for GLA was very prominent in liver lipid spectra 12–24 h after dosing with [3-^{13}C] GLA but then decayed rapidly and disappeared by 96 h post-dosing (Fig. 2B). The NMR signal for C-5 of liver AA at 128.9 ppm was highest at 48 h and was quite prominent from 24 to 96 h post-dosing (Fig. 2A). Hence the peak ^{13}C enrichment in liver AA occurred at a time when enrichment in ^{13}C in liver GLA was almost back to baseline values (Fig. 4).

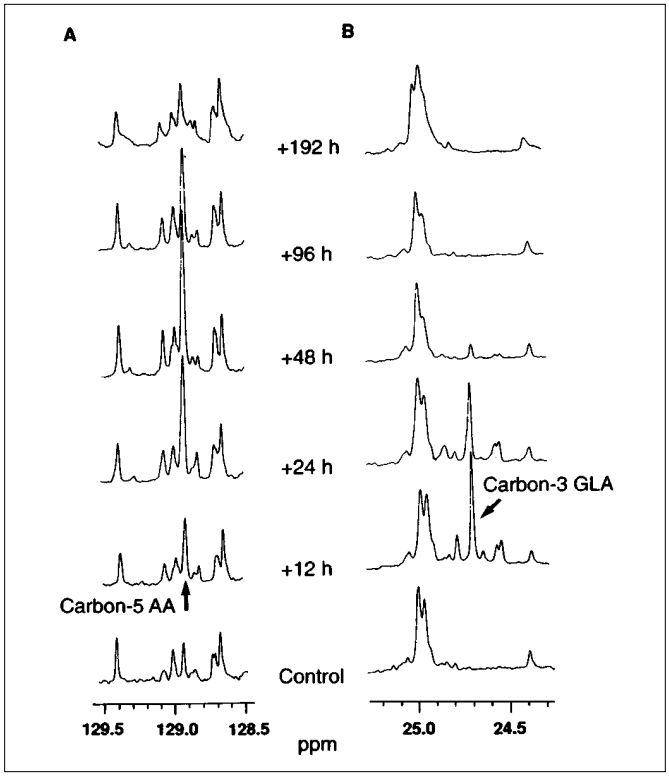

FIG. 2. The time course of ^{13}C enrichment at carbon (C)-5 of arachidonic acid (A) and C-3 of γ-linolenic acid (B) in ^{13}C nuclear magnetic resonance spectra of liver total lipid extracts. Suckling rats were dosed with [3-^{13}C]-γ-linolenic acid on day 6 and the time course lasted to day 14.

Other ^{13}C-enriched NMR signals in the spectra of liver lipids occurred in the region immediately surrounding C-3 of GLA (24.5–24.9 ppm). These signals have not yet been identified due to a lack of appropriate standards, but they may arise from C-3 of GLA esterified to different lipid classes or to GLA at different positions on phospholipid or triglyceride molecules. NMR-detectable ^{13}C enrichment was not readily identified with C-5 of dihomo-γ-linolenic acid or C-7 of adrenic acid (22:4n-6) in liver lipids because these resonances are not well-resolved among many other aliphatic carbons between 29–30 ppm.

In ^{13}C NMR spectra of brain total lipids, no ^{13}C enrichment was detected at any time point at the resonance of C-3 of GLA (Fig. 3B). However, C-5 of AA (128.9 ppm) was enriched with a maximum peak 48 h after dosing with the [3-^{13}C] GLA (Fig. 3A).

Experiment 1: IRMS of liver and brain total lipids. In Experiment 1, the atom % of ^{13}C-GLA in liver phospholipids rose within 48 h to 2–3 times natural abundance levels but for ^{13}C-AA, the ^{13}C enrichment only increased 9% above natural abundance levels (Table 1). However, relative to the total GLA or AA pool in liver phospholipids, total mass of ^{13}C enrichment in AA was 7 times higher than in GLA at 24 h and 16 times higher at 48 h post-dosing (Table 1). In liver triglycerides, ^{13}C enrichment in GLA reached 3–4 atom % and the

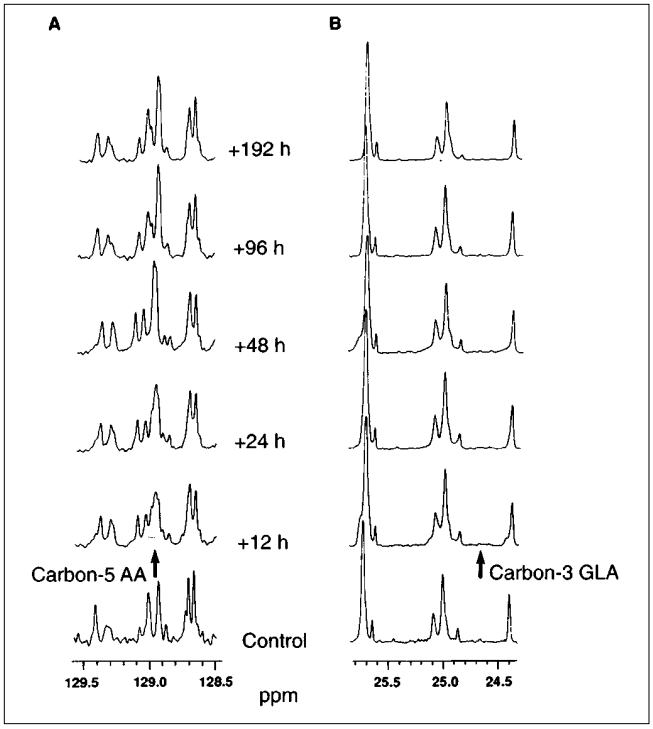

FIG. 3. The time course of ^{13}C enrichment at carbon-5 of arachidonic acid (A) and carbon-3 of γ-linolenic acid (B) in ^{13}C nuclear magnetic resonance spectra of brain total lipid extracts. Suckling rats were dosed with [3-^{13}C]-γ-linolenic acid on day 6 and the time course lasted to day 14.

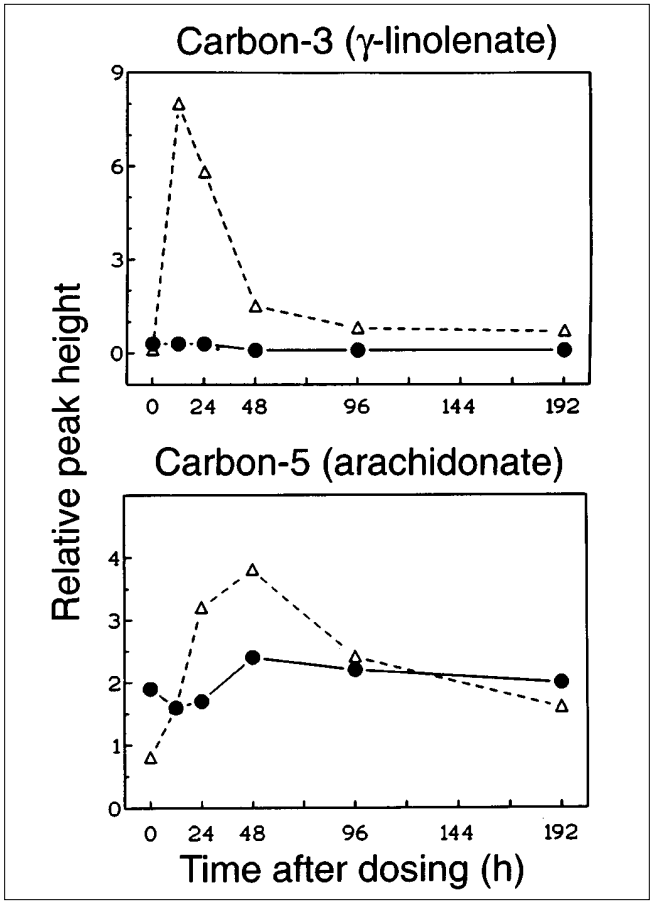

FIG. 4. Peak height for carbon (C)-3 of γ-linolenic acid and carbon (C)-5 of arachidonic acid in ^{13}C nuclear magnetic resonance spectra of total lipids of liver (△--△) and brain (●—●) from suckling rats after dosing with [3-^{13}C]-γ-linolenic acid. Peak heights are shown relative to peak for the n-3 carbons of n-3 polyunsaturated fatty acids (132 ppm) which cannot be enriched with ^{13}C.

peak mass of ^{13}C in GLA did not exceed 30% of that in AA (data not shown). In brain phospholipids, peak ^{13}C enrichment occurred 48–96 h post-dosing and the atom % ^{13}C-GLA rose by 5–14%; the atom % increase in ^{13}C-AA was about 4% (Table 2). High ^{13}C enrichment was detected 48 h in GLA of brain free fatty acids but ^{13}C mass in this pool was not determined. Relative to the total pools of GLA and AA in brain phospholipids, ^{13}C mass enrichment in AA was 44 and 130 times higher than in GLA at the 48 h and 96 h points, respectively (Table 2).

In brain total lipids, ^{13}C enrichment peaked at 48 h post-dosing and plateaued near peak levels between 96–192 h (Fig. 5A). The ^{13}C enrichment curve for brain phospholipids (Fig. 5B) was similar to that for C-5 of AA (Fig. 4) suggesting the ^{13}C mass enrichment of AA accounted for most of the ^{13}C in brain phospholipids. Since brain weight and total lipid content increased over the study period (Fig. 6), the plateau

TABLE 1
Peak Levels of ^{13}C Enrichment in γ-Linolenic Acid (GLA) and Arachidonic Acid (AA) of Liver Phospholipids[a]

		GLA	AA
Atom % excess	+24 h	2092[b]	126
(× 10^{-3})	+48 h	845	116
^{13}C Mass[c]	+24 h	+85	+600
	+48 h	+34	+561

[a]From 7–8-day-old suckling rats after oral dosing with 20 mg [3-^{13}C] GLA (Experiment 1).
[b]Mean of 3–4 samples/time point; samples were pooled before analysis.
[c]ng ^{13}C/g tissue, based on ^{13}C at a average natural abundance of 1.09560% in GLA and AA, 5 μg/g GLA and 600 μg/g AA in liver phospholipids at 7–8 days old (Ref. 21).

TABLE 2
Peak Levels of ^{13}C Enrichment in γ-Linolenic Acid (GLA) and Arachidonic Acid (AA) of Brain Phospholipids[a]

		GLA	AA
Atom % excess	+48 h	209[b]	47
(× 10^{-3})	+96 h	64	48
^{13}C mass[c]	+48 h	+3	+131
	+96 h	+1	+132

[a]From 8–10-day-old suckling rats after oral dosing with 20 mg of [3-^{13}C] GLA (Experiment 1).
[b]Mean of 3–4 samples/time point; samples were pooled before analysis.
[c]ng ^{13}C/g tissue, based on ^{13}C at a natural abundance of 1.09560% in GLA and AA, and on 2 μg/g GLA and 350 μg/g AA in brain phospholipids at 8–10 days old (Ref. 21).

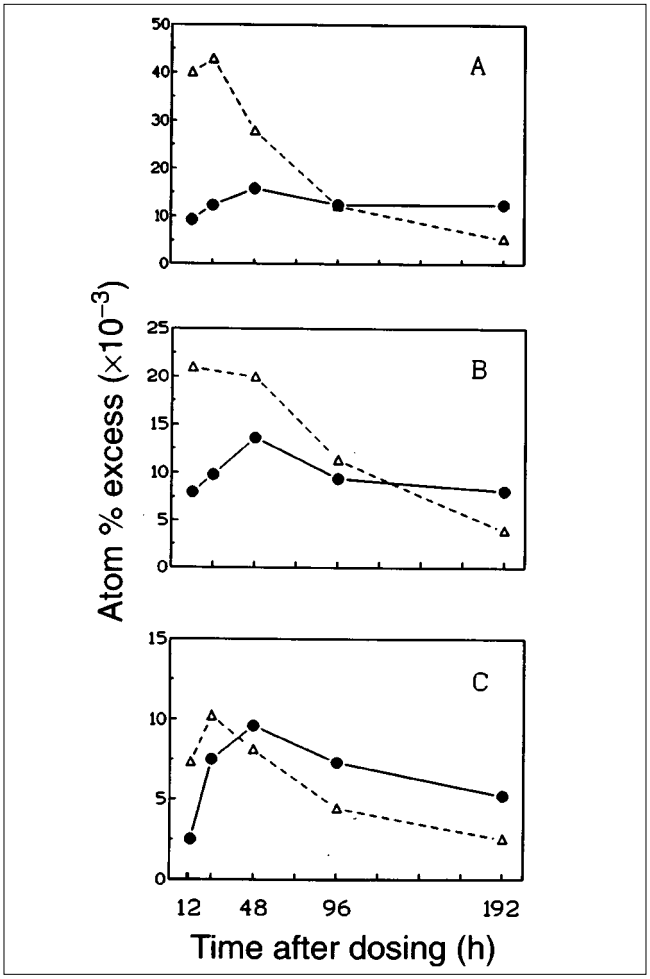

FIG. 5. The time course of ^{13}C enrichment (atom % excess) in rat brain (●—●) and liver (△– –△) total lipids (A), phospholipids (B), and free cholesterol (C) after dosing with [3-^{13}C] γ-linolenic acid. Suckling rats were dosed at day 6, and the time course lasted to day 14. Each point represents data from 3–4 tissue samples which were pooled together before being analyzed in duplicate.

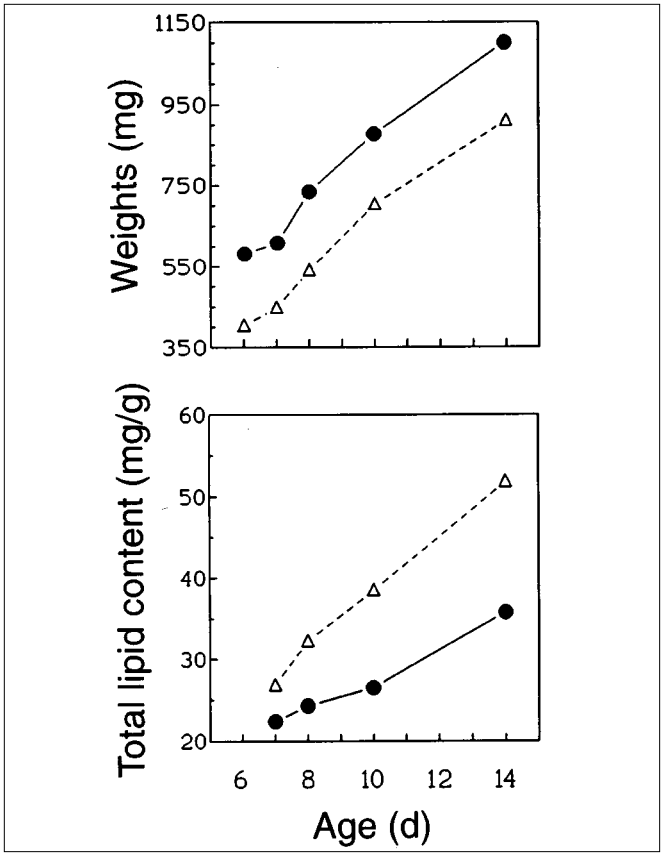

FIG. 6. Brain (●—●) and liver (△– –△) weights, and total lipid content of suckling rats.

in ^{13}C APE in brain total lipids indicated that ^{13}C mass in brain total lipids increased in parallel with increasing brain weight. ^{13}C enrichment in brain lipids increased faster in cholesterol than in total phospholipids but peaked in both lipid classes about 48 h after dosing with [3-^{13}C] GLA (Fig. 5B,C). In liver total lipids, ^{13}C APE peaked 24 h post-dosing and then decreased to 192 h (Fig. 5A). Since liver weight and lipid content increased over the study (Fig. 6) while ^{13}C enrichment decreased, net mobilization of ^{13}C-enriched lipids from the liver must have occurred over the course of the study. ^{13}C APE in liver phospholipids was about twice as high as in liver free cholesterol (Fig. 5B,C).

Experiment 1: water-soluble metabolites. ^{13}C enrichment in water-soluble metabolites peaked in the brain 2 h after dosing, peaked in the liver 2–6 h after dosing, and peaked in the remaining carcass 2 h after dosing with [3-^{13}C] GLA (Fig. 7). Maximal ^{13}C enrichment was similar in water-soluble metabolites of liver, brain, and carcass at about 20×10^{-3} APE.

Experiment 2: lower dose ^{13}C-GLA. The separation of liver and brain fatty acids by GC prior to ^{13}C analysis by GC–C–IRMS showed that ^{13}C enrichment was present in several n-6 PUFA derived from GLA (Table 3) but was not present in other fatty acids including palmitic, stearic, oleic, linoleic, or in n-3 PUFA (data not shown).

TABLE 3
^{13}C Enrichment (atom % enrichment × 10^{-3}) in γ-Linolenic Acid (GLA), Dihomo-γ-Linolenic Acid (DGLA), Arachidonic Acid (AA), and Adrenic Acid (ARA; 22:4n-6) of Liver and Brain Phospholipids[a]

	GLA	DGLA	AA	ARA
Liver				
+48 h	377 ± 242[b]	77 ± 3	9 ± 1	9 ± 1
+96 h	274 ± 310	65 ± 51	14 ± 10	8 ± 5
Brain				
+48 h	40 ± 33	29 ± 14	11 ± 2	7 ± 1
+96 h	66 ± 1	15 ± 10	11 ± 8	8 ± 4

[a]From 8–10-day-old suckling rats after oral dosing with 5 mg [3-^{13}C] GLA (Experiment 2).
[b]Mean ± SD of 2–4 samples/time point; samples were pooled after analysis.

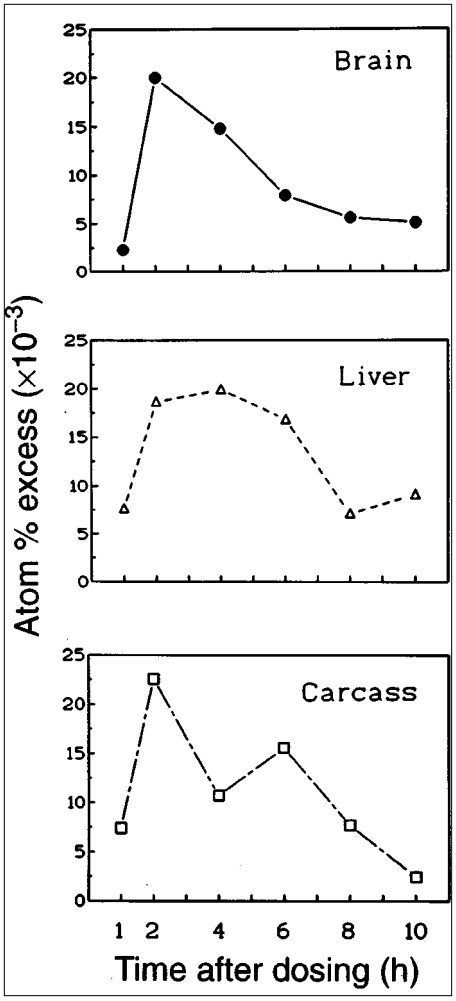

FIG. 7. The time course of ^{13}C enrichment in water-soluble extracts of (A) brain (●—●), (B) liver (△– –△), and remaining (C) carcass (□— – —□) of 6-day-old suckling rats dosed with [3-^{13}C]-γ-linolenic acid. Each point represents data from 3–4 tissue samples which were pooled together before being analyzed in duplicate.

DISCUSSION

The present results show that a single tracer dose of [3-^{13}C]-GLA given by a physiological route is readily detected by ^{13}C-NMR of extracted liver lipids, but only a low mass of GLA itself accessed the lipids of the suckling rat brain within 96 h of dosing. By the time that ^{13}C levels in liver GLA had nearly returned to baseline (48 h), ^{13}C enrichment in AA had peaked in both liver and brain lipids (Fig. 4). Most ^{13}C-AA in the brain peaked at about the same time as in the liver. Data acquired by GC–C–IRMS clearly showed that ^{13}C was present in GLA in brain free fatty acids and that some [3-^{13}C]-GLA entered brain phospholipids. In view of the small pool of GLA in total brain lipids, the mass of [3-^{13}C]-GLA entering brain phospholipids within 96 h of providing a high dose of tracer (20 mg) was very low and equivalent to about 1–2% of the mass of ^{13}C-labeled AA in brain phospholipids (Table 2).

Combined with the relatively low brain uptake of ^{13}C-GLA itself, these data suggest that AA in the brain is derived primarily from ^{13}C-AA synthesized outside the brain, probably mostly in the liver; the ^{13}C NMR data (Fig. 3) and the GC–C–IRMS (Fig. 5, Table 2) as well as other studies in the literature (18) are in agreement on this point. In the context of similar work done using cultured neurons and astrocytes (19), it appears that if some conversion of [3-^{13}C]-GLA to [5-^{13}C]-AA occurred in the brain, it would probably have happened in astrocytes.

In addition to GLA, some linoleic acid also accesses the brain of the suckling rat (20), so it appears that the developing rat brain can derive AA either directly from the diet (maternal milk at the age studied here), from AA stores in adipose tissue, or from synthesis inside or outside the brain. Lean tissue triacylglycerols are also rich in PUFA including AA in the suckling period (21). Without AA in the diet, the adequacy of its endogenous synthesis remains in question and probably depends on maturity at birth, nutritional status, age of study, and species. The species difference or the difference in maturity at birth is evident when the present data are compared to those previously reported for the piglet, in which essentially no ^{13}C-AA was detectable by NMR in brain lipids after dosing with ^{13}C-GLA (8). With the caveat that measuring levels of ^{13}C in the whole brain may mask more rapid utilization of GLA for AA synthesis by some cell types in the brain (19), the present results indicate that the low levels of GLA in the mammalian brain are probably due to relatively low uptake of GLA by the brain and are less likely to be due to rapid conversion of dietary GLA to AA in the brain itself.

We did not evaluate oxidation of ^{13}C-GLA in this study but Leyton *et al.* (22) have shown that about 30% of a single bolus dose of [1-^{14}C]-GLA given to adult rats is oxidized to $^{14}CO_2$ within 24 h of dosing which is considerably less than for linoleic or α-linolenic acids. Thus, once formed, GLA seems less prone to oxidation than other 18-carbon PUFA. Nevertheless, our data on ^{13}C recovered in water-soluble metabolites (Fig. 7) and in brain and liver cholesterol (Fig. 5) indicate that some GLA was oxidized and that ^{13}C was recycled into lipids synthesized *de novo*. This route of metabolism of PUFA carbon into lipids synthesized *de novo* has previously been demonstrated for linoleic (23) and for α-linolenic acids (17). Keto acids such as acetoacetate and β-hydroxybutyrate are important lipogenic substrates in suckling rats (24,25) and probably constitute the main ^{13}C-labeled metabolites in the water-soluble extracts we prepared (26).

We conclude that in suckling rats, a low amount of exogenous GLA has direct access to brain lipids and that most brain AA derived from exogenous GLA is probably synthesized elsewhere first. This study only addressed the utilization of GLA, and so the proportion of brain AA which is derived *via* synthesis compared to that which may have been transported to the brain from adipose tissue or maternal milk cannot be determined from our data. Whether dietary GLA may be a

more efficient precursor to brain AA if the suckling rats were receiving no dietary AA was also not addressed in the present study. This would require artificial rearing using a formulated semipurified liquid diet to control the exact PUFA type and intake. Our ongoing research is addressing the applicability of ^{13}C-GLA as an *in vivo* probe for noninvasive NMR studies.

ACKNOWLEDGMENTS

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An Esterification Protocol for *cis*-Parinaric Acid-Determined Lipid Peroxidation in Immune Cells[1,2]

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ABSTRACT: Loss of fluorescence from *cis*-parinaric acid (cPnA) is a sensitive indicator of lipid peroxidation. The purpose of this study was to utilize cPnA to determine, at the level of the intact immune cell, whether enrichment of membranes with polyunsaturated fatty acids (PUFA) increased lipid peroxidation. P388D1 macrophages were labeled by addition of cPnA as an ethanolic solution. Within two minutes of addition, in the absence of serum, cPnA rapidly intercalated into the plasma membrane. Lipid peroxidation was initiated by addition of Fe^{2+}-EDTA resulting in a dose-dependent decrease in fluorescence with increased oxidant concentration. Cells previously enriched with PUFA and labeled by intercalation showed no differences in spontaneous or Fe^{2+}-induced lipid peroxidation. In separate experiments, 20 μM cPnA in ethanolic solution was injected into cell culture media containing 0.1% essentially fatty acid free bovine serum albumin (BSA). Cells were resuspended and incubated for 90 min at 37°C. After washing with BSA to remove cPnA which had not incorporated, 0.5% (0.1 μM) of the added cPnA was found esterified within cellular lipids. This level of cPnA provided a 100-fold increase over basal autofluorescence levels. Cells labeled in this manner also lost fluorescence in a dose-dependent manner as levels of oxidant stress increased. Cells enriched with PUFA and labeled by esterification had significantly increased rates and total amounts of lipid peroxidation. Co-incubation with α-tocopherol and PUFA resulted in a decrease in lipid peroxidation which was not significantly different from control cells. In conclusion, esterification of cPnA into membrane phospholipids can sensitively detect changes in lipid peroxidation induced by alteration of membrane PUFA and/or vitamin E content.

Lipids 32, 219–226 (1997).

Membrane fatty acid composition can be rapidly modified by changing the source of dietary lipid (1,2). In fact, when polyunsaturated oils are increased in the diet as a means of decreasing saturated fat, tissues become enriched with polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA). Membranes containing increased levels of these fatty acids are more easily oxidized (3,4). Indeed, increased lipid peroxidation has been observed in rats (5–9) and humans (10,11) upon feeding fish oil which is rich in EPA and DHA. Tissue damage resulting from lipid peroxidation has been implicated in conditions such as atherosclerosis, sickle cell anemia, hemochromatosis, ischemia-reperfusion injury, and arthritis (12,13).

Vitamin E is widely accepted to be the primary lipid-soluble antioxidant responsible for protection of unsaturated membrane components. Increasing dietary PUFA has been reported to be antagonistic to vitamin E status (14–17). Both the antagonistic effect of dietary PUFA and the proposed increased requirement at the level of the PUFA-enriched membrane have provided a basis for an increase in the dietary vitamin E requirement (16–19). Increases in membrane unsaturation without concomitant increases in membrane vitamin E could render cells more susceptible to oxidative stress. Macrophages, which produce reactive oxygen species (e.g., $O_2^{-\cdot}$, H_2O_2) for phagocytic killing, could be at potentially greater risk than other cells and tissues (20,21). Consequently, the ability to measure lipid peroxidation at the level of the intact cell becomes important in the determination of an overall dietary vitamin E requirement.

Although numerous methods are available for measuring lipid peroxidation, these methods are discontinuous and indirect, requiring time point sampling. Oxidative by-products are monitored rather than the actual, continuous process of lipid peroxidation. These methods require tissue homogenization and sample preparation which may include lipid extraction, denaturing of protein and chemical derivatization, possibly introducing artifacts (13,22,23). Recently, a new method has been described which allows direct measurement of lipid peroxidation at the level of the cell membrane without disruption of cellular integrity and function (24–26). This sensitive method which is both direct and continuous, uses *cis*-parinaric acid (9, 11, 13, 15-*cis*-*trans*-*trans*-*cis*-octadecatetraenoic acid, cPnA), a naturally occurring fatty acid with conjugated tetraene structure which renders it fluorescent in a

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Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; cPnA, *cis*-parinaric acid, 9, 11, 13, 15-*cis*-*trans*-*trans*-*cis*-octadecatetraenoic acid; DHA, docosahexaenoic aicd; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; PBS, phosphate buffered saline; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography; UV, ultraviolet.

lipid environment. Exposure of erythrocyte ghosts or intact erythrocytes to ethanolic cPnA results in rapid intercalation of the free fatty acid into the membrane bilayer. Rearrangement of the tetraene structure of cPnA after electron abstraction during lipid peroxidation causes loss of fluorescence and is therefore a direct measure of lipid peroxidation which may be monitored as it occurs.

One of the overall goals of our laboratory is to understand the interaction between dietary PUFA and vitamin E, relative to lipid peroxidation, at the level of the immune cell. Our objective was to use cPnA in intact immune cells to assess the effect of modulating membrane components (i.e., PUFA and α-tocopherol), on the susceptibility of living cells to lipid peroxidation. Here, we present evidence that with minor modifications to the published method, useful information about nutritional modulation of lipid peroxidation in immune cells can be obtained.

MATERIALS AND METHODS

Materials. Solvents were high-performance liquid chromatography grade or Fisher Optima grade (Fisher Scientific Co. St. Louis, MO). Silica gel thin-layer chromatography (TLC) plates were purchased from Fisher Scientific Co. (St. Louis, MO). α-Tocopherol was the kind gift of Eastman Kodak Chemical (Rochester, NY). Arachidonic acid (AA) and eicosapentaenoic acid (EPA) were purchased from Cayman Chemical Co. (Ann Arbor, MI). cPnA was purchased from Molecular Probes Inc. (Plano, TX). Stock solutions of cPnA (2 μM in study #1, 20 μM in study #2), α-tocopherol, and fatty acids were made in absolute ethanol, previously deoxygenated by bubbling with nitrogen gas. Stock solutions were stored at −80°C under nitrogen gas. The murine monocyte cell line, P388D1, was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cell culture reagents were obtained from the Cell and Immunobiology Core Facilities (University of Missouri, Columbia, MO).

Cell culture. P388D1 cells were maintained in RPMI 1640 supplemented with 15% fetal bovine serum (FBS), 1 mM glutamine, 100 μg/mL penicillin, and 100 units/mL streptomycin (complete medium). Cells were grown in 75 cm^2 flasks with confluent cells subcultured after detaching by mild scraping.

Enrichment of P388D1 cells with α-tocopherol and/or AA and EPA. For all experiments involving cellular enrichment with α-tocopherol or the polyunsaturated fatty acids (PUFA), AA and EPA, stock solutions in absolute ethanol were added to complete media which was then used to seed cells grown to near confluence (90%). At no time did total ethanol addition exceed 0.2% of the total volume. Incorporation of these membrane constituents was promoted by allowing cells to incubate at 37°C for 12 h under a humidified atmosphere of 95% air and 5% CO_2. After this culture period, cells were harvested by gentle scraping as previously described. Addition of an equal concentration of absolute ethanol into complete media was used in control cell cultures. Viability of cells, as determined by trypan blue exclusion, exceeded 90% under all culture conditions described.

Intercalation of fluorescent probe in cellular membranes. P388D1 cells were found to have a low level of autofluorescence (less than four arbitrary fluorescence units) at 324 and 413 nm for excitation and emission, respectively. Injection of 2 μM cPnA into the cell suspension resulted in a dramatic and immediate rise in fluorescence (i.e., 400-fold increase). In the presence of a 30% filter, fluorescence stabilized within two minutes and was determined to be a linear function of probe concentration between 1 and 4 μM cPnA. A cell concentration of 4×10^5 cells/mL was sufficient to completely incorporate 4 μM cPnA such that cPnA remaining in the water phase was minimal. Therefore for our studies with the monocyte cell line, we chose injection of 2 μM ethanolic cPnA solution into a cell suspension containing 4 to 6×10^5 cells/mL of phosphate buffered saline (PBS) without Ca^{+2} and Mg^{+2} followed by a two-minute equilibration period for the intercalation experiments. Each compound used in the assay (e.g., ethanol, $FeCl_2 \cdot 4\ H_2O$, ethylenediaminetetraacetate, vitamin E) was tested individually to check for autofluorescence and/or quenching of the cPnA signal prior to establishing individual protocols.

Incorporation of fluorescent probe into cellular lipids. The labeling protocol for esterification experiments was established as 20 μM cPnA in 0.1% essentially fatty acid-free bovine serum albumin (BSA) in RPMI 1640 for 90 min. The increase in cPnA concentration was necessary to provide a 100-fold increase in fluorescent signal over the basal autofluorescence. cPnA in ethanolic solution was injected into complete media which was then used to resuspend cells for incubation. After a 90-min incubation at 37°C, cells were washed three times with RPMI 1640 containing albumin (0.1% BSA), which was sufficient to remove nonesterified cPnA from the cells. The amount of cPnA not incorporated into the cells was determined by separate extraction of the lipids in the original supernatant and each of the three wash supernatants with 4 vol of chloroform/methanol (2:1, vol/vol). The cPnA in each fraction was quantified by ultraviolet (UV) absorbance (304 nm) after the sample was resuspended in absolute ethanol.

The incorporation of cPnA into the cellular lipids of P388D1 was assessed by extracting total lipids from cell pellets. Briefly, 4 vol of chloroform/methanol (2:1, vol/vol) were mixed with the thawed cell pellet. The lower phase was placed into a glass tube and evaporated in a 40°C water bath under a stream of N_2 gas. The total lipid extract was resuspended in a small volume of chloroform/methanol (2:1, vol/vol), and applied to a 2-cm lane of a TLC plate (27). Total cellular lipids were separated into major lipid classes (i.e., phospholipid, triglyceride, cholesterol ester, free cholesterol, free fatty acids) using hexane/diethyl ether/glacial acetic acid (80:20:2, by vol) as the developing solvent (28). Identity of lipid classes was verified with commercial standards. Individual lipid classes were removed from the plate by scraping and then extracted with 750 μL double-distilled H_2O and 3 mL chloroform/methanol (2:1, vol/vol). Individual lipid classes

were resuspended in absolute ethanol and the cPnA concentration determined by UV absorbance at 304 nm.

In order to provide gas chromatographic verification that cPnA was esterified into membrane phospholipids, it was necessary to modify the labeling conditions. P388D1 cells were harvested at 90% confluence and pelleted by centrifugation at $250 \times g$ for 10 min. Cells were resuspended at a concentration of 10^6 cells/mL in complete media to which 40 μM cPnA had been added then incubated. After a 24-h incubation at 37°C, cells were harvested and cellular lipids were extracted using the methods described previously. Lipid classes (i.e., phospholipid, free fatty acid, triglyceride, cholesterol ester) were separated as previously described. Isolated lipids were transmethylated with 4% H_2SO_4 in methanol (88°C, 1 h). Some cell lipid extracts were subjected to two-dimensional TLC in order to determine the incorporation of cPnA into individual phospholipid classes. In this case, methyl esters were prepared by transmethylation using 0.5M NaOH in methanol (28). Fatty acid methyl esters were extracted and then injected into a Hewlett-Packard gas chromatograph (Sunnyvale, CA) Model 5890A with a 30-m capillary column (Supelcowax 10; Supelco, Bellefonte, PA). Results, expressed as the percentage of total fatty acids, were determined by electronic integration (Hewlett-Packard 3380A integrator).

Lipid peroxidation assay. Two mL of cells in PBS (0.4 to 0.6×10^6 cells/mL), which had been labeled with cPnA either by intercalation or esterification, were placed in each of four cuvettes in a Perkin-Elmer spectrofluorometer MPF-66 (Norwalk, CT), equipped with a stirring apparatus and a jacketed, automated four-chamber cuvette holder to maintain the cells at 37°C for the time course of the experiment. Fluorescence was monitored by excitation at 324 nm and determining emission at 413 nm (29). Intercalation experiments were conducted in the presence of a 30% light filter. After measurement of initial fluorescence from each cuvette, lipid peroxidation was initiated by addition of an equimolar solution of $FeCl_2 \cdot 4H_2O$ and EDTA such that final concentrations were 10 and 100 μM Fe^{+2} in 10 and 100 μM EDTA, respectively (30). Lipid peroxidation has been shown to be initiated by hydroxyl radicals derived from a Fenton-type reaction when ferrous iron and EDTA are present in equimolar concentrations (30–32). Loss of fluorescence, which served as a measure of lipid peroxidation, was monitored simultaneously every 2 min for each of four cuvettes, in real time and is expressed as the percentage of initial fluorescence remaining at a given time point. Since cPnA fluorescence has been found to undergo spontaneous decay after exposure to light, a cuvette containing cPnA-labeled cells with no additives or added oxidant stress was monitored simultaneously as a control for each experiment (24). The rate of spontaneous decay of cPnA signal in control cells was measured with and without enrichment with α-tocopherol. The presence of this antioxidant did not change either the rate or amount of fluorescence lost, indicating that loss of signal in control cells was probably due to photobleaching rather than lipid peroxidation of the probe.

Statistical analysis. All data were analyzed as a repeated, split-plot analysis of variance as outlined by Gil and Hafs (33). The linear statistical model contained the effect of treatment, replicate within treatment, time and treatment × time (34,35). The main effect of treatment was tested using replicate within treatment as the denominator of F. All trend analyses were tested using the residual error term. Each experiment was run in duplicate with values averaged for each treatment. Each experiment was replicated from three to six times as indicated in the figure legends. A one-degree-of-freedom polynomial linear and quadratic contrast was used for testing trend analyses (36).

RESULTS

Location of esterified label. To determine the quantity and location of cPnA esterified into cellular lipids, a series of experiments were conducted utilizing the UV properties of this fatty acid. This approach is more sensitive than gas chromatography. After 90 min incubation with 20 μM cPnA in the presence of 0.1% essentially fatty acid free BSA, the incorporation of cPnA into cellular lipids was less than 1% (0.6%) of the added cPnA. In order to ensure that the fluorescent signal was coming from esterified rather than intercalated cPnA, cells were washed with RPMI which contained 0.1% essentially fatty acid-free BSA to facilitate removal of any cPnA intercalated into the plasma membrane. Total lipids from wash supernatants were extracted and the cPnA concentration determined. Over 98% of the cPnA originally added was recovered in the incubation supernatant with less than 1% recovered in the first wash and less than 0.2% in each of the subsequent two washes. Results from five separate experiments showed that under these conditions, approximately 50% of the incorporated cPnA was found in the phospholipids, 20% in the triglycerides, 18% in the cholesterol esters, and 12% in the free fatty acid fraction. Although less than 1% of the added cPnA was esterified into cellular lipids (0.1 μM), this amount was sufficient to provide an adequate fluorescence with which to conduct these studies. Furthermore, the majority of the cPnA was located in the membrane phospholipid fraction of these cells.

Chromatographic evidence for cPnA incorporation into phospholipid is provided in Figure 1 and was accomplished by increasing both the concentration of cPnA and the length of incubation time. The peak identified as cPnA was present in the TLC isolated phospholipid fraction of cells incubated with cPnA, but was absent in cells incubated without exogenous cPnA. The cPnA peak in enriched cells eluted at the same time as did the methyl ester of the free fatty acid. Separation of P388D1 phospholipid classes by two-dimensional TLC gave evidence that cPnA was in the phosphatidylcholine and phosphatidylethanolamine fractions. In contrast, when P388D1 cells were incubated with cPnA in medium without a source of albumin (e.g., no FBS), incorporation of cPnA into phospholipids was not observed (data not shown).

The effect of labeling method on the sensitivity of cPnA to increasing levels of oxidant stress. P388D1 cells were labeled

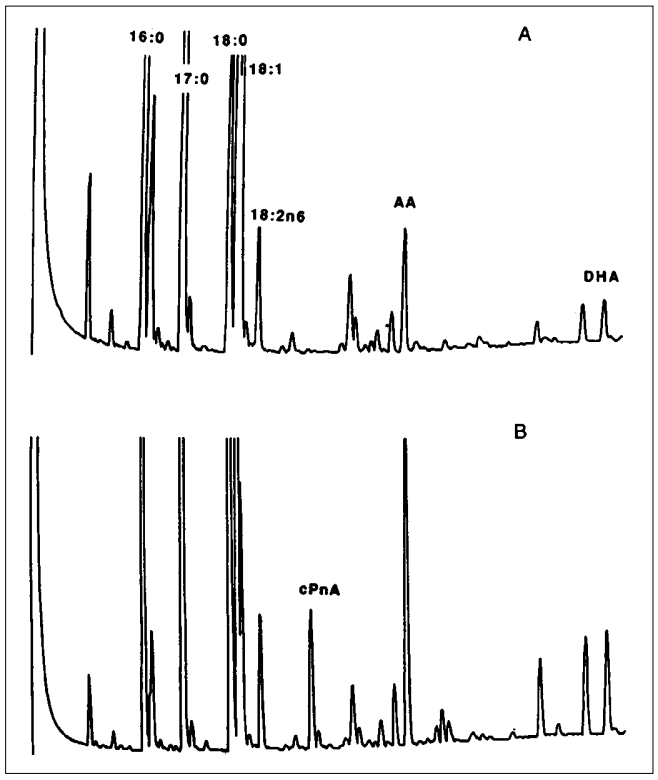

FIG. 1. Gas chromatogram of phospholipid extracted from P388D1 cells and separated by one-dimensional thin-layer chromatography, incubated without (A) or with (B) 40 μM *cis*-parinaric acid, 9, 11, 13, 15-*cis-trans-trans-cis*-octadecatetraenoic acid (cPnA) in the presence of 0.1% essentially fatty acid-free bovine serum albumin for 24 h; DHA, docosahexaenoic acid; AA, arachidonic acid.

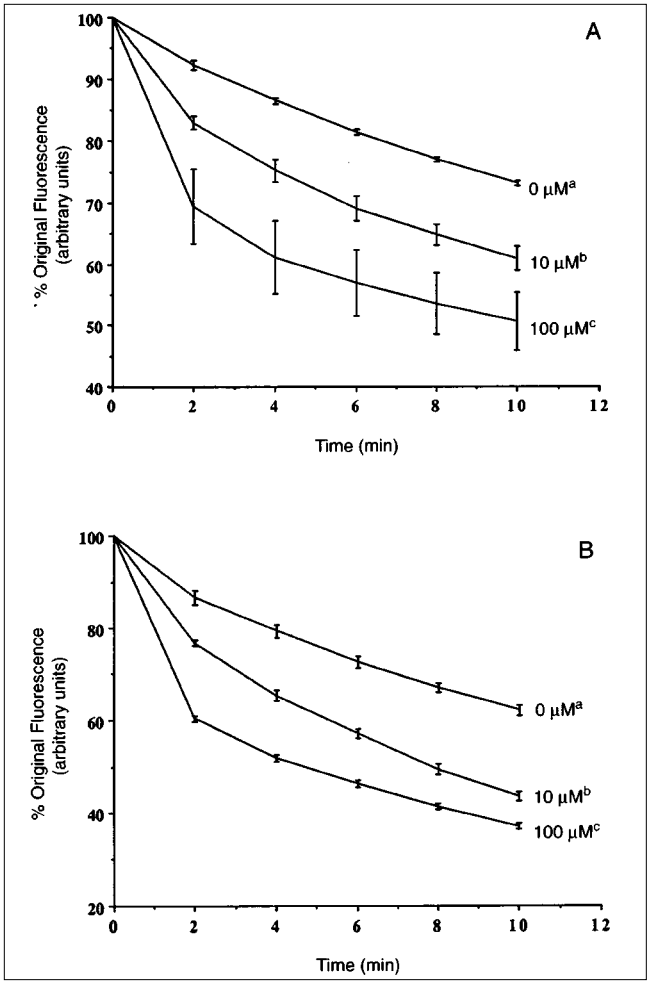

FIG. 2. (A) Effect of increasing oxidant stress on loss of fluorescence in P388D1 cells, labeled with 2 μM cPnA by intercalation for two minutes. Cell concentration was 0.5×10^6 cells/mL in phosphate-buffered saline without calcium or magnesium. Lipid peroxidation was induced at time zero by addition of 10 or 100 μM $FeCl_2 \cdot 4\ H_2O$ in equimolar EDTA. Zero μM represents spontaneous decay of signal from control cells in the presence of the light source. Values used are means ± SEM for three separate experiments. Curves assigned different superscript letters are significantly different ($P < 0.05$) based on linear and quadratic analysis. (B) Effect of increasing oxidant stress on loss of fluorescence in P388D1 cells, labeled by esterification with 20 μM cPnA in 0.1% bovine serum albumin for 90 min. Cell concentration was 0.5×10^6 cells/mL in phosphate-buffered saline without calcium or magnesium. Lipid peroxidation was induced at time zero by addition of 10 or 100 μM $FeCl_2 \cdot 4\ H_2O$ in equimolar EDTA. Zero μM represents spontaneous decay of signal from control cells in the presence of the light source. Values used are means ± SEM for three separate experiments. Curves assigned different superscript letters are significantly different ($P < 0.05$) based on linear analysis. Quadratic analysis shows that the shapes of the curves for the 0 and 100 μM treatment groups were not different from each other, but were significantly different from those exposed to 10 μM $FeCl_2$ ($P < 0.05$). See Figure 1 for abbreviations.

by the intercalation method, and then subjected to increasing levels of oxidative stress (i.e., Fe^{2+}-EDTA complex). The rate at which fluorescence was lost increased in a dose-dependent fashion (Fig. 2A). Both the rate at which lipid peroxidation occurred and the total amount of probe oxidized were significantly affected by treatment as evidenced by significant differences in linear and quadratic function ($P < 0.05$). Cells labeled by esterification of cPnA (Fig. 2B) responded in a similar manner to increasing levels of oxidant stress as provided by ferrous-EDTA. Each increasing dose of ferrous-EDTA resulted in a significant increase in both rate and total fluorescence lost ($P < 0.05$). Cells labeled by esterification lost from 13 to 17% more fluorescence (10 and 100 μM Fe^{2+}–EDTA complex, respectively) than did cells labeled by intercalation. When differences in the kinetics of photobleaching were taken into consideration, this difference was reduced to only 5%.

Oxidant stress provided by enrichment with long-chain PUFA. To further evaluate the use of cPnA as a means to assess lipid peroxidation in immune cells, we incubated P388D1 cells in media supplemented with two long-chain PUFA (i.e., 5 μM EPA plus 5 μM AA). After 12 h, the degree of membrane unsaturation was greatly increased. This was primarily a result of the replacement of linoleic acid with EPA and AA in membrane phospholipids. For example, the AA content of the phosphatidylcholine fraction from these cells was increased from 0.915 to 2.08 nmoles/10^6 cells and EPA from 0.0 to 0.41 nmoles/10^6 cells for EPA with a concomitant

30% decrease in linoleic acid. We hypothesized that such an enrichment in unsaturation would lead to significantly greater loss of cPnA upon oxidative challenge. However, the increased levels of long-chain PUFA did not increase the loss of basal fluorescence or loss of signal in the presence of an oxidant stress when cells were labeled by the intercalation method (data not shown). In contrast, upon exposure to the light source, without addition of our exogenous oxidant stress, cells enriched with PUFA and then labeled by esterification were found to have a significantly greater lipid peroxidation (i.e., loss of fluorescence) as compared to control cells

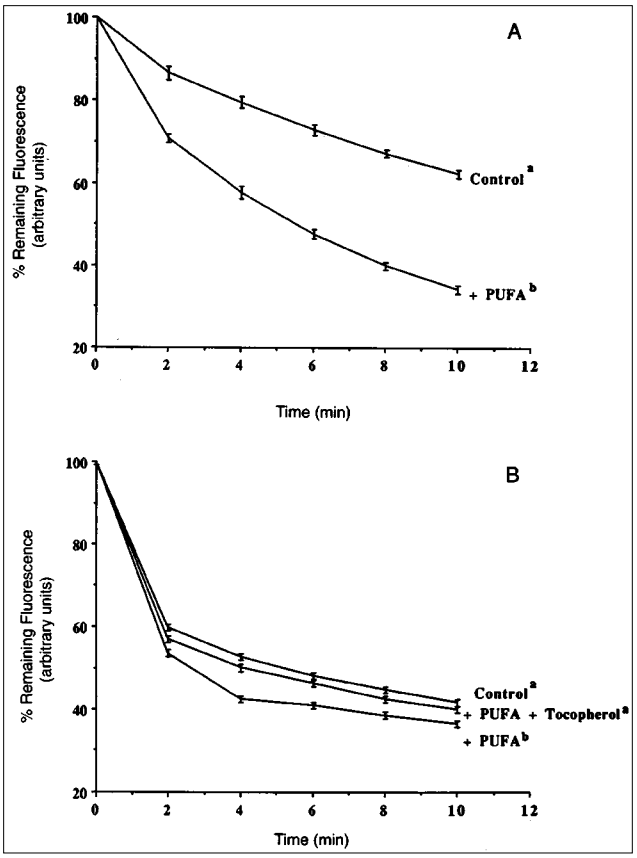

FIG. 3. (A) Effect of incubation with or without 5 μM arachidonic acid (AA) and 5 μM eicosapentaenoic acid on the spontaneous loss of fluorescent signal in P388D1 cells, labeled by esterification with 20 μM cPnA in 0.1% bovine serum albumin for 90 min. Cell concentration was 0.5×10^6 cells/ mL in phosphate-buffered saline. Values used are means ± SEM for three separate experiments. Curves assigned different superscript letters are significantly different based on linear and quadratic analysis ($P < 0.05$). (B) Effect of incubation with or without 5 μM arachidonic and 5 μM eicosapentaenoic acid and 250 μM d-α-tocopherol for 12 h on the loss of fluorescent signal in P388D1 cells, labeled by esterification with 20 μM cPnA in 0.1% bovine serum albumin for 90 min. Cell concentration was 0.5×10^6 cells/ mL in phosphate-buffered saline. Lipid peroxidation was induced at time zero by addition of 10 μM $FeCl_2 \cdot 4\ H_2O$ in equimolar EDTA. Values used are means ± SEM for six separate experiments. Curves assigned different superscript letters are significantly different based on linear and quadratic analysis ($P < 0.05$); PUFA, polyunsaturated fatty acids. See Figure 1 for other abbreviations.

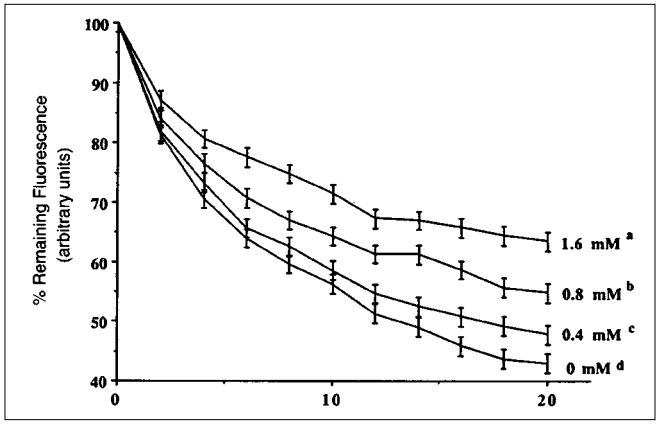

FIG. 4. Effect of incubation with increasing concentrations of d-α-tocopherol: 0.0, 0.4, 0.8, or 1.6 mM for 12 h, on the loss of fluorescence in P388D1 cells, labeled by esterification with 20 μM *cis*-parinaric acid, 9, 11, 13, 15-*cis-trans-trans-cis*-octadecatetraenoic acid (cPnA) in 0.1% bovine serum albumin for 90 min. Cell concentration was 0.5×10^6 cells/mL in PBS. Lipid peroxidation was induced at time zero by addition of 100 μM $FeCl_2 \cdot 4\ H_2O$ in equimolar EDTA. Values used are least square means ± SEM for three separate experiments. Curves assigned different superscript letters are significantly different based on linear analysis ($P < 0.05$). Quadratic analysis shows that the shapes of the curves from the 0.0 and 0.4 mM additions of d-α-tocopherol were significantly different from that of the 1.4 mM d-α-tocopherol, but not from the 0.8 mM d-α-tocopherol.

(Fig. 3A). When these cells were exposed to an additional oxidant stress, the PUFA-enriched cells lost significantly more fluorescence than did control cells. The addition of 250 μM α-tocopherol in the incubation media along with the long-chain PUFA, prevented the increase in lipid peroxidation noted when cells were incubated with PUFA alone (Fig. 3B).

Protection against lipid peroxidation by α-tocopherol. Cells were enriched with vitamin E by incubating them in complete media to which 250 μM α-tocopherol had been added. Control cells were incubated in complete media without the addition of α-tocopherol. After 12 h, control and α-tocopherol-enriched cells were split and labeled by intercalation and by esterification, then subjected to different levels of oxidant stress. At either level of oxidative stress, loss of fluorescence in the cells labeled by intercalation was not significantly affected by overnight incubation of cells in tocopherol-enriched media. In contrast, cells enriched with α-tocopherol and labeled by esterification of cPnA lost significantly less fluorescence than did control cells ($P < 0.05$, data not shown). The effect of increasing α-tocopherol concentration in the incubation media from 0, 0.4, 0.8, and 1.2 mM for 12 h on lipid peroxidation induced by 100 μM ferrous-EDTA is shown in Figure 4. Although cells labeled by intercalation showed no differences in lipid peroxidation due to increasing concentrations of vitamin E (data not shown), when cells were labeled by esterification, vitamin E significantly reduced the rate at which fluorescence was lost, in a dose-dependent fashion ($P < 0.05$).

DISCUSSION

One of the overall goals of our laboratory is to understand the interaction between dietary PUFA, particularly fish oil, and vitamin E. Increases in dietary PUFA are rapidly reflected in the type of fatty acids found in the membrane. Since part of the suggested increase in dietary requirement of vitamin E is based on increased lipid peroxidation in membranes enriched with PUFA, it was particularly relevant for us to be able to examine lipid peroxidation at the level of the immune cell.

Our initial objective was to use the published method of intercalating cPnA into intact immune cells to assess the effects of modulation of membrane components (i.e., PUFA and α-tocopherol) on the susceptibility of living cells to lipid peroxidation. The intercalation of cPnA as a method for monitoring lipid peroxidation, originally published for use in liposomes (24), has been successfully adapted for use in erythrocyte ghosts (25,37,38), sarcoplasmic reticulum vesicles (39,40), intact erythrocytes (25,26,41,42), and Chinese hamster ovary cells (43). Tsuchiya and coworkers (44) were able to utilize cPnA and a model membrane system (i.e., liposomes) to characterize the antioxidant properties of α-tocopherol, β-carotene, and ubiquinol in response to a lipid-soluble, free radical initiation system. In an attempt to adapt the method to immune cells, we chose to work with the murine monocyte cell line P388D1, with which we have experience modulating the cellular content of both α-tocopherol and PUFA.

We observed increased lipid peroxidation in response to increased levels of oxidant stress provided by chelated ferrous iron. When ferrous iron and EDTA are present in equimolar concentrations, lipid peroxidation is initiated by hydroxyl radicals. Cells labeled with cPnA by intercalation and esterification showed increased rate and total amount of peroxidation as the oxidant stress was increased from 10 to 100 μM Fe-EDTA. These data parallel the effect of *in vivo* administration of ferrous iron. For example, Hu and coworkers (7) found a positive correlation between the concentration of ferrous iron injected intraperitoneally into rats and indices of lipid peroxidation in various tissues such as serum, liver, and kidney. In comparing both methods of labeling our cells, we observed that the esterified cPnA label was slightly more sensitive to the same level of oxidant stress than cells labeled by intercalation. When corrected for basal loss of fluorescence, the 13 to 17% increased sensitivity observed in the esterified cells is reduced to a 5% difference. Although we have characterized the loss of cPnA fluorescence in untreated cells as photobleaching because it was not altered by exogenous vitamin E, it is also possible that this is a background oxidation. Greater oxidation of cPnA in intercalated cells where the label is closer to the surface of the cell and therefore the interface of cell to media may alter the kinetics of background loss of fluorescence due to lipid peroxidation not initiated by the experimenter. Since the goal of these experiments was to investigate whether we could show greater susceptibility of PUFA-enriched cells toward oxidation relative to cells with lower concentrations of PUFA, we chose not to pursue the question of differences between methods.

Using the intercalation method, we were not able to detect increases in lipid peroxidation due to membrane PUFA enrichment. No significant differences in loss of basal fluorescence or loss of fluorescence in the presence of an exogenous oxidant stress were found in the cells PUFA-enriched vs. nonenriched cells labeled with cPnA by intercalation. Our data are in agreement with those of Van den Berg and coworkers (26) who reported that cPnA intercalated into intact erythrocytes was not able to detect increased lipid peroxidation associated with membrane PUFA enrichment. However, in that study erythrocytes from fish oil-fed rabbits lost significantly more total membrane PUFA as determined by lipid extraction and gas chromatographic analysis than those from control rabbits. To our knowledge, this is the first report of lipid peroxidation determined by cPnA that did not have a strong positive correlation with other methods of lipid peroxidation that were run simultaneously. In the direct measurement of lipid peroxidation of submitochondrial particles, de Hingh and coworkers (45) found excellent positive correlation between values determined by cPnA and oxygen consumption. We concluded that although intercalated cPnA works well as an indicator of lipid peroxidation in most cases, it does not reflect increased lipid peroxidation due to PUFA enrichment.

Rather than abandoning the protocol, we examined the potential for cPnA incorporation into membrane phospholipids. We hypothesized that altering the membrane location such that cPnA was incorporated as a normal membrane component would allow greater sensitivity in detection of lipid peroxidation. Previously, Chinese hamster ovary cells have been reported to be capable of incorporating exogenously supplied cPnA into their membrane phospholipids. That cPnA was provided with a source of albumin such as fetal calf serum was essential (28). Sklar and Dratz (46) reported similar incorporation into bovine retinal rod outer segments. Harris and Stahl (47) suggested that the uptake of cPnA into phospholipids is an enzymatic process mediated by acyl-CoA acyltransferase and have shown its uptake is competitively inhibited by oleic acid. They further report that 71% of the esterified cPnA was found in phosphatidylcholine and 20% in phosphatidylethanolamine. Stuhne-Sekalec *et al.* (48), using guinea pig liver microsomes, have shown that cPnA is preferentially esterified at the *sn*-2 position although small quantities were found at the *sn*-1 position. Further, they showed that cPnA could be readily cleaved by phospholipase A_2. Several authors have also shown that as a naturally occurring fatty acid, cPnA can be bound to cellular lipid transfer proteins such as fatty acid binding protein and sterol carrier protein (49) and can be esterified into cellular phospholipids (50). Prows and coworkers (52) have demonstrated that uptake of cPnA into the plasma membrane in the absence of serum is very rapid. Further, the total quantity of cPnA incorporated was significantly increased when fibroblasts were transfected with liver fatty acid binding protein. Although this technique provides a ready source of cPnA for incorporation into mem-

branes, less than 3% of cPnA was esterified after a 30-min incubation. Our experience with P388D1 cells showed that they could rapidly esterify PUFA into membrane phospholipids when these fatty acids were introduced as fatty acid–albumin complexes. It must be noted, however, that only 0.5% of the total cPnA was esterified in 90 min in contrast to the rapid uptake of 2 μM cPnA when no albumin is present. Therefore, although cPnA can be esterified into mammalian cells, it must be emphasized that the total amount is very small. Also, the cPnA that is incorporated by esterification is distributed throughout all cellular membranes (43) rather than concentrated into the plasma membrane.

The idea of esterifying the fluorescent probe to the membrane phospholipid was a particularly appealing one. Researchers have proposed that membrane α-tocopherol is physically associated with certain membrane phospholipids (52–56). Therefore, esterification of cPnA into the *sn*-2 position of the membrane phospholipid renders the cPnA in proper orientation for interaction with membrane α-tocopherol. Thus, this approach provides a physiologically relevant model for monitoring lipid peroxidation within cell membranes and the effect of alterations in membrane phospholipid PUFA and α-tocopherol on this process. We believe that cPnA orientation during intercalation may result in a lack of proximity among intercalated cPnA, membrane phospholipids, and vitamin E and that these factors are important determinants for success in examining differences in lipid peroxidation due to alterations of cell membrane components.

In conclusion, we have shown that esterification of low concentration of cPnA (<0.1 μM) into membrane phospholipids provides a sensitive real time method for observing relative differences in rate and amount of oxidation as a function of immune cell PUFA and vitamin E content. This method allows direct monitoring of the process of lipid peroxidation as it is occurring. It requires no processing of samples such as homogenization or lipid extraction and should therefore be subject to less artifactual error. We propose that it may be useful as a means for *in situ* examination of the initial stages of lipid peroxidation and their modulation by membrane components in isolated immune cells.

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Novel Oxylipins from the Temperate Red Alga *Polyneura latissima*: Evidence for an Arachidonate 9(*S*)-Lipoxygenase

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ABSTRACT: The oxylipin chemistry of the temperate red alga *Polyneura latissima* has been investigated. The structures of three novel oxylipins, 8-[1′(*Z*),3′(*Z*),6′(*Z*)-dodecatriene-1′-oxy]-5(*Z*),7(*E*)-octadienoic acid, 7(*S**)-hydroxy-8(*S**),9(*S**)-epoxy-5(*Z*),11(*Z*),14(*Z*)-eicosatrienoic acid, 7(*R**)-hydroxy-8(*S**),9(*S**)-epoxy-5(*Z*),11(*Z*),14(*Z*)-eicosatrienoic acid, together with two known eicosanoids, 9(*S*)-hydroxy-5(*Z*),7(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid, and 9,15-dihydroxy-5(*Z*),7(*E*),11(*Z*),13(*E*)-eicosatetraenoic acid, were elucidated by spectroscopic methods and chemical degradation. The oxygenation pattern of these oxylipins suggests that *P. latissima* metabolizes polyunsaturated fatty acids *via* a 9(*S*)-lipoxygenase.

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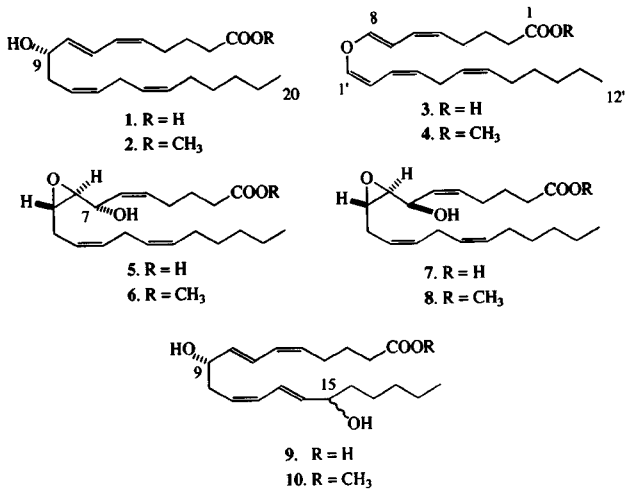
It is well established in mammalian tissues that arachidonic acid can be oxygenated at different positions by lipoxygenases of varying positional specificity, and that this leads to a wide array of physiologically and pathologically important eicosanoids (e.g., HETE, hepoxilins, and leukotrienes). Interestingly, several marine algae have yielded eicosanoids that are identical to mammalian metabolites (1–3). However, our continued exploration of new oxylipins from diverse marine algae has led to the discovery of a number of structurally unique compounds that are without parallel in mammals (4,5).

The red alga *Polyneura latissima* is widely distributed along the Pacific coast of North America from Alaska to Mexico in the lower intertidal and subtidal zones to a depth of 10–20 meters. The initial analysis of a *P. latissima* crude extract from northern California indicated that this red alga contained eicosanoids different from those previously isolated from marine plants. The promising fractions produced by initial chromatography were treated with diazomethane and then further purified by additional column chromatography and high-performance liquid chromatography (HPLC) to provide the pure methyl ester derivatives.



The northern California collection of *P. latissima* provided compounds **2**, **6**, **8,** and **10** as well as a very small amount of compound **4**. A second collection made from the state of Washington provided an additional amount (7.1 mg) of compound **4** and enabled completion of its structural elucidation. The structures of these methyl ester derivatives were developed from consideration of various spectroscopic data, particularly two-dimensional nuclear magnetic resonance (NMR), in concert with chemical degradations.

EXPERIMENTAL PROCEDURES

Instruments. Infrared (IR) spectra were recorded on Nicolet 5 DXB FT 15 and Nicolet 510 spectrophotometers, (Madison, WI). NMR spectra were recorded on Bruker AC 300 and AM 400 spectrometers (Karlsruhe, Germany) with trimethylsilyl (TMS) as internal standard. Finnigan 4023 (San Jose, CA) and Kratos MS 50TC (Ramsey, NJ) spectrometers were used to obtain low resolution (LR) mass spectral (MS) resolution and high resolutions (HR) MS. Gas chromatography–mass

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Abbreviations: COSY, correlation spectroscopy; diHETE, dihydroxyeicosatetraenoic acid; GC–MS, gas chromatography–mass spectrometry; HETCOR, heteronuclear correlation; HETE, hydroxyeicosatetraenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; HR EIMS, high-resolution electron impact mass spectrometry; IR, infrared; LR, low resolution; MS, mass spectral; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, trimethylsilyl; UV, ultraviolet.

spectrometry (GC–MS) was carried out with a Hewlett-Packard Model 5970B mass selective detector (Palo Alto, CA) connected to a Hewlett-Packard Model 5890 gas chromatograph. HPLC was performed using a Waters M-6000 pump (Milford, MA) and U6K injector. Optical rotation was measured on a Perkin-Elmer 141 polarimeter (Norwalk, CT). Thin-layer chromatography (TLC) used Merck (Darmstadt, Germany) aluminum-backed TLC sheets (silica gel 60 F_{254}). All solvents were distilled prior to use.

Collection, extraction, and isolation. The first collection of *P. latissima* was made from the intertidal zone at Duxberry Reef on the California coast on June 22, 1989. The alga was immediately frozen with CO_2 (s) after collection and stored frozen until work-up. The defrosted alga (0.3 kg dry weight) was homogenized in $CHCl_3$/MeOH (2:1, vol/vol), warmed for 15 min, and then filtered. The chloroform and water layers were separated, and the former was evaporated *in vacuo* to give 0.37 g of dark green oil. The extract was fractionated by silica gel chromatography in the vacuum mode using a gradient of EtOAc in hexanes. The fraction eluting with 15% EtOAc/hexanes contained trace amounts of compound **4**. The materials eluting with 30% EtOAc/hexanes were methylated with diazomethane. The methylated materials were further purified by vacuum chromatography to give compound **2** (14.4 mg, 3.9% yield of the crude extract). The materials eluting with 50% EtOAc/hexanes were also methylated with diazomethane and fractionated over silica gel. The compounds eluting with 15–30% EtOAc/hexanes were further isolated by HPLC to give compound **6** (1.5 mg, 0.4% yield of the crude extract), compound **8** (2.6 mg, 0.7% yield of crude extract), and compound **10** (0.5 mg, 0.14% yield of the crude extract.

A second collection of the alga was made from the Puget Sound area, Washington. The seaweed (90 g dry weight) gave 2.6 g of crude extract. Fractionation of 2.4 g of this extract in a manner similar to that described above gave an additional 7.1 mg of compound **4** (0.3% yield).

*Compound **2***. A colorless oil; $[\alpha]_D^{23} = +11.1^0$ (c = 0.56, MeOH); ultraviolet (UV) (MeOH) λ_{max} = 236 nm (ε = 21,500); IR (neat) 3450, 3011, 2953, 2927, 2858, 1737, 1439, 1210, 1163, 987, 723 cm^{-1}; TMS-ether electron impact mass spectrometry (EI MS) *m/z* (rel. int. %) 406 (0.4), 391 (2), 375 (0.6), 316 (1), 285 (0.8), 255 (99.4), 226 (13.9), 181 (8), 151 (22), 133 (31), 105 (45), 73 (100).

*Steric analysis of compound **2***. A small amount of compound **2** was ozonized for 12 min in 1 mL $CHCl_3$ at –20°C. The volume of the solution was reduced under N_2. The menthoxycarbonyl derivative was formed in a solution of 50 µL toluene, 50 µL menthoxychlorocarbonate, and 10 µL pyridine for 30 min at room temperature The reaction mixture was partitioned 3× between hexane and H_2O. The hexane solubles were treated with peracetic acid at 50°C overnight, reduced under argon, dissolved in MeOH, and then treated with CH_2N_2. The methylated material was purified *via* preparative TLC (25% EtOAc/hexanes), eluted with Et_2O, and analyzed by GC–MS vs. standards. The steric composition at C-9 was found to be 93% *S* and 7% *R*.

*Compound **4***. A colorless oil; UV (MeOH) λ_{max} = 268 (ε = 32,000), 206 (ε = 21,000); IR (neat) 2954, 2931, 2870, 1736, 1440, 1376, 1164, 1124, 1073, 972 cm^{-1}; LR EIMS *m/z* (rel. int. %) 333 (0.7), 332 (3), 266 (0.7), 221 (0.8), 189 (2), 179 (2), 169 (1), 170 (2), 163 (2), 153 (3), 137 (6), 109 (9), 96 (14), 91 (16), 84 (10), 43 (100); HR EIMS *m/z* obs. $[M]^+$ 332.2351 ($C_{21}H_{32}O_3$, 0.0 mmu dev.).

*Compound **6***. A colorless oil; $[\alpha]_D^{23} = +70°$ (c = 0.07, $CHCl_3$); IR (neat) 3448, 3091, 2926, 2860, 1735, 1651, 1544, 1450, 1371, 1241, 1155, 1036, 732, 680 cm^{-1}; LR EIMS *m/z* (rel. int. %) 332 (0.3, $M^+ - H_2O$), 319 (0.3), 303 (0.4), 290 (1.4), 231 (1.4), 215 (1.7), 193 (4), 170 (7), 157 (24), 125 (37), 95 (41), 79 (75), 67 (65), 55 (82); HR EIMS *m/z* obs. $[M + H]^+$ 351.2500 ($C_{21}H_{35}O_4$, –3.5 mmu deviation), 333.2430 ($[M + H]^+ - H_2O$, $C_{21}H_{33}O_4$, 0.0 mmu deviation).

*Compound **8***. A colorless oil; $[\alpha]_D^{23} = -58°$ (c = 0.19, $CHCl_3$); IR (neat) 3448, 3010, 2927, 2859, 1735, 1652, 1445, 1369, 1317, 1213, 1161, 1123, 1084, 1024, 943, 893, 868, 766, 717 cm^{-1}; LR EIMS *m/z* (rel. int. %) 332 (0.3, $M^+ - H_2O$), 319 (0.3), 303 (0.5), 290 (1.4), 231 (1.4), 215 (1.7), 193 (4), 170 (8), 157 (22), 125 (38), 95 (41), 79 (77), 67 (67), 55 (81).

*Compound **10***. A colorless oil; $[\alpha]_D^{23} = -7.5°$ (c = 0.05, MeOH); UV (MeOH) λ_{max} = 238 nm (ε = 49,600), 232 nm (ε = 47,700), 204 (ε = 18,000); IR (neat) 3406, 2951, 2929, 2860, 1735, 1438, 1247, 1215, 1164, 987, 952 cm^{-1}; LR EIMS of TMS-ether derivative of **10** *m/z* (rel. int. %) 422 (0.03), 373 (0.5), 329 (0.4), 313 (0.7), 255 (100), 239 (3), 223 (4), 181 (5), 165 (12), 151 (13), 133 (21), 123 (12), 105 (21), 73 (64).

RESULTS AND DISCUSSION

Compound **2**, the most abundant eicosanoid metabolite of *P. latissima,* showed $[\alpha]_D^{23} = +11.1^0$ (c = 0.56, MeOH). Its ^1H NMR spectrum was quite similar to that of methyl 12-HETE, the only difference being the shape of multiproton signals between δ5.4 and δ5.5 (6). The HETE nature of this compound was clearly visible by ^1H-^1H correlation spectroscopy (COSY) in which the olefinic proton at δ5.71 of the *cis-trans* conjugated diene (10.9 and 15.2 Hz coupling) was spin-coupled with an α-hydroxy proton at δ4.22. Nevertheless, the position of oxidation in this compound was not clear from NMR data alone because of overlapping signals between δ5.4 and δ5.5. The mass spectrum of the TMS-ether derivative of compound **2** yielded a molecular ion at *m/z* 406 with crucial fragments at *m/z* 255 and 151 (cleavage of C-9–C-10) which placed the hydroxyl group at C-9. By analysis of ^1H-^1H coupling constants and ^{13}C NMR chemical shifts, compound **2** was identified as methyl-9-hydroxy-5(*Z*),7(*E*),11(*Z*),14(*Z*)-eicosatetraenoate. Previously, 9-HETE has been observed from human psoriatic skin (7) and has been chemically synthesized (8,9). The stereochemistry of compound **2** was determined by GC–MS analysis of a menthoxycarbonyl derivative of dimethylmalate which was produced from **2** by ozone degradation and methylation, and showed that C-9 in compound **2** was predominantly of *S* configuration (see the Experimental Procedures section).

Compound **4** was a colorless oil and had UV absorption at 268 and 206 nm. HR EIMS gave its molecular formula as $C_{21}H_{32}O_3$ in which 6° of unsaturation could be easily as-

signed by ^{13}C NMR analysis to one ester group and five double bonds. By ^{1}H-^{1}H COSY and ^{1}H-^{13}C heteronuclear correlation (HETCOR) spectra, two highly polarized double bonds (δ106.61and δ147.91; δ105.54 and δ142.10) were shown to be components of two separate spin systems, one consisting of 12 carbon atoms (C-1′ to C-12′) and the other of 7 carbon atoms (C-2 to C-8). In addition to the carbomethoxy group (Table 1), the partial structures given above represented all of

TABLE 1
^{1}H and ^{13}C Data for Methyl Ester Derivatives 2, 4, 6, 8, and 10[a]

	Compound **2**[b]				Compound **6**[b]				Compound **8**[b]			
	^{1}H NMR			^{13}C NMR	^{1}H NMR			^{13}C NMR	^{1}H NMR			^{13}C NMR
C#	δ	*m*	*J*(Hz)	δ	δ	*m*	*J*(Hz)	δ	δ	*m*	*J*(Hz)	δ
1				—[c]				173.94				173.90
2	2.32	*t*	7.4	33.32	2.34	*t*	7.4	33.25	2.33	*t*	7.4	33.25
3	1.72	*p*	7.4	24.72	1.75	*q*	7.3	24.64	1.73	*q*	7.4	24.60
4	2.23	*dq*	1.0, 7.4	26.99	2.19	*m*		27.16	2.16	*m*		27.16
5	5.42	*m*		131.74	5.62	*m*		133.73	5.58	*m*		133.03
6	6.02	*t*	10.9	128.75	5.42	*m*		128.18	5.56	*m*		128.68
7	6.51	*tdd*	0.9, 11.0, 15.2	125.54	4.62	*m*		64.87	4.30	*m*		67.35
8	5.71	*dd*	6.4, 15.2	135.53	2.86	*dd*	2.4, 5.4	59.61	2.85	*dd*	2.2, 5.0	60.75
9	4.22	*d*	6.2	71.98	3.06	*dt*	2.4, 5.4	54.07	3.00	*dt*	2.2, 5.4	55.86
10	2.36	*m*		35.34	2.43	*m*		29.29	2.43	*m*		29.29
					2.31				2.32			
11	5.44	*m*		124.58	5.39	*m*		123.14	5.40	*m*		123.06
12	5.51	*m*		131.23	5.32	*m*		127.14	5.35	*m*		127.11
13	2.81	*t*	7.0	25.81	2.78	*t*	7.1	25.74	2.78	*t*	7.1	25.74
14	5.34	*m*		127.24	5.55	*m*		131.50	5.55	*m*		131.51
15	5.40	*m*		130.67	5.36	*m*		130.75	5.45	*m*		130.75
16	2.04	*q*	6.8	27.22	2.04	*q*	6.6	27.24	2.02	*q*	7.0	27.23
17	1.21–1.37[e]	*m*		29.28	1.25–1.37[e]	*m*		29.29	1.21–1.38[e]	*m*		29.29
18	1.21–1.37	*m*		31.49	1.25–1.37	*m*		31.51	1.21–1.38	*m*		31.50
19	1.21–1.37	*m*		22.56	1.25–1.37	*m*		22.58	1.21–1.38	*m*		22.58
20	0.88	*t*	6.7	14.07	0.89	*t*	6.8	14.08	0.89	*t*	6.8	14.07
MeO	3.67	*s*		51.53	3.68	*s*		51.61	3.67	*s*		51.61

	Compound **10**[b]					Compound **4**[b]			
	^{1}H NMR			^{13}C NMR		^{1}H NMR			^{13}C NMR
C#	δ	*m*	*J*(Hz)	δ	C#	δ	*m*	*J*(Hz)	δ
1				—[c]	1				174.04
2	2.33	*t*	7.4	3.33	2	2.33	*t*	7.4	33.32
3	1.72	*p*	7.4	24.72	3	1.72	*p*	7.4	24.74
4	2.23	*q*	7.4	27.01	4	2.15	*dp*	1.1, 7.4	26.94
5	5.44	*m*		131.46	5	5.27	*m*		128.03
6	6.02	*t*	11.0	128.71	6	5.89	*dt*	1.1, 11.7	124.19
7	6.50	*m*		125.76	7	6.08	*t*	11.7	106.61
8	5.72	*dd*	4.3, 15.0	135.29	8	6.60	*d*	11.7	147.91
9	4.26	*q*	6.2	72.03	1′	6.22	*d*	6.1	142.10
10	2.47	*dd*	7.3, 11.8	35.76	2′	5.53	*ddd*	0.9, 6.1, 11.1	105.54
					3′	6.36	*dd*	1.0, 11.1	120.29
11	5.46	*m*		130.91	4′	5.38	*m*		129.27
12	6.16	*t*	10.9	126.87	5′	2.88	*t*	7.1	26.03
13	6.49	*m*		125.28	6′	5.36	*m*		127.07
14	5.73	*dd*	4.7, 15.2	137.39	7′	5.42	*m*		130.77
15	4.17	*q*	6.4	72.76	8′	2.05	*q*	6.8	27.21
16	1.54	*m*		37.26	9′	1.26–1.38[e]	*m*		29.28
17	1.25–1.40[e]	*m*		24.72[d]	10′	1.26–1.38	*m*		31.49
18	1.25–1.40	*m*		25.10[d]	11′	1.26–1.38	*m*		22.55
19	1.25–1.40	*m*		22.60	12′	0.89	*t*	6.8	14.07
20	0.88	*t*	6.5	14.06	MeO	3.67	*s*		51.50
MeO	3.67	*s*		51.57					

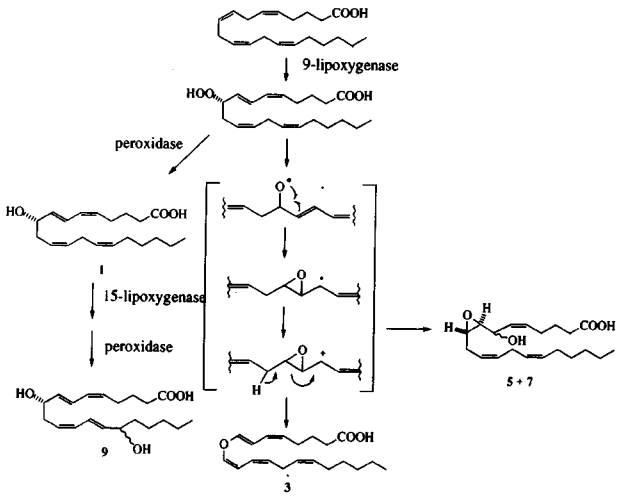
[a]^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz and ^{13}C NMR spectram at 75 MHz in $CDCl_3$ with trimethylsilyl as an internal chemical shift reference.
[b]All ^{13}C NMR signals were assigned by heteronuclear correlation experiments.
[c]Not observed.
[d]Assignments exchangeable.
[e]Overlapped signals not assigned.

the atoms in the molecule except for one oxygen atom which must therefore connect the two spin systems. Furthermore, the coupling constants of 11.7 Hz and 6.1 Hz for the two pairs of olefinic protons on the two polarized double bonds were diagnostic for their stereochemistry (C-7,8 is *E* and C-1′,2′ is *Z*). Vinyl ether compounds, such as ethyl propenyl ether (10) and colneleic acid (11), exhibit coupling constants of 6–7 Hz between *cis* olefinic protons and 11–12 Hz between *trans* protons. This combination of data allowed deduction of structure **4** as methyl 8-[1′(*Z*),3′(*Z*),6′(*Z*)-dodecatrienyloxy]-5(*Z*),7(*E*)-octadienoate. The corresponding natural product, 8-[1′(*Z*),3′(*Z*),6′(*Z*)-dodecatrienyloxy]-5(*Z*),7(*E*)-octadienoic acid (**3**), is a new substance in nature to which we assign the trivial name "polyneuric acid."

In the 1970s, Galliard and coworkers (11–13) reported on the discovery of the unusual divinyl ethers colneleic acid and colnelenic acid from potato tubers. Later, Corey *et al.* (14) synthesized colneleic acid and found that this molecule was an effective inhibitor of potato lipoxygenase. Fahlstadius and Hamberg (15) showed that enzymatic removal of the *pro-R* hydrogen from C-8 of 9*S*-hydroperoxyoctadecadienoic acid was involved in colneleic acid biosynthesis. Polyneuric acid (**3**) is structurally similar to colneleic acid and colnelenic acid but differs in chain length and the geometry of one of the two double bonds attached to the bridging oxygen atom.

From HR and LR EIMS, compounds **6** and **8** had the same molecular formula of $C_{21}H_{34}O_4$ and almost identical MS fragmentation patterns. Their structural similarity was also revealed in their ^{1}H NMR spectra where the only significant difference was the chemical shift of a proton geminal to a hydroxyl group (Table 1). By ^{13}C NMR data, four of the five degrees of unsaturation inherent in their molecular formulas were attributable to three double bonds and one carbomethoxy group. In the ^{1}H-^{1}H COSY of compound **8**, the proton at δ4.30 was correlated to a midfield proton at δ 2.85 which was further coupled to another midfield proton at δ3.00 by 2.2 Hz, suggestive of an epoxide. This relationship of functional groups, which compound **6** possessed as well, was reminiscent of a hepoxilin (16). From the ^{1}H-^{13}C HETCOR data for compound **8**, the protons at δ2.85 and δ3.00 were correlated to carbons at δ60.75 and δ55.86, respectively, thereby confirming the epoxide functionality. Having accounted for all of the degrees of unsaturation, functional groups and heteroatoms in **6** or **8**, it only remained to assign the location of oxidation and stereochemistry in these two metabolites.

The position of the epoxy-alcohol functionality in compounds **6** and **8** was identified by the LR EIMS fragmentation pattern (fragment masses *m/z* 157 and 193 result from cleavage of C7–C8 in both **6** and **8**)(see the experimental Procedures section). The stereochemistry of the hydroxyl groups relative to the epoxy functionality was determined by comparing the chemical shifts and coupling patterns of the α-hydroxyl protons of both compounds to model compounds (17,18). The α-hydroxyl proton of the *threo*-isomer is consistently at higher field than that of the *erythreo*-isomer (17). The epoxide for each compound was shown to be *trans* by virtue of a 2.2–2.4 Hz coupling between the epoxide protons. Although double-bond



SCHEME 1

geometries could not be identified by conventional coupling constant analysis, they were effectively revealed by the ^{13}C NMR chemical shifts of the carbons vicinal to the olefins (19,20). A carbon vicinal to a *trans* double bond shows a chemical shift 4–6 ppm downfield as compared to that vicinal to a *cis* double bond (21). Analysis of ^{13}C NMR shifts for compounds **6** and **8** showed all double bonds to be of *cis* configuration. By these data, compound **6** was identified as methyl 7(S^*)-hydroxy-8(S^*),9(S^*)-epoxy-5(*Z*),11(*Z*),14(*Z*) eicosatrienoate and compound **8** was identified as methyl 7(R^*) -hydroxy-8(S^*),9(S^*)- epoxy-5(*Z*),11(*Z*),14(*Z*) eicosatrienoate.

Compound **10**, a colorless oil, showed $[\alpha]_D^{23} = -7.5°$ (c = 0.05, MeOH). The ^{1}H-^{1}H COSY of **10** showed that the α-hydroxyl protons (δ4.26 and δ4.17) correlated to two overlapping conjugated systems [λ_{max} = 238 nm (ε = 49,600), 232 nm (ε = 47,700)]. Despite this degeneracy, it was still possible by very careful analysis to map out the proton connectivity from the C-2 methylene (δ2.33) to the C-20 methyl group (δ0.88). The coupling constants of the olefinic protons were clarified by proton-decoupling experiments; each diene was of the expected *cis-trans* configuration. Additional support for the location of oxidation in **10** was obtained from LR EIMS of the TMS-ether derivative which showed ions at *m/z* 255 (cleavage of C9–C10) and *m/z* 239 (cleavage of C15–C16)(see the Experimental Procedures section). Compound **10** was thus identified as methyl 9(S^*),15-dihydroxy 5(*Z*),7(*E*),11(*Z*),13(*E*)-eicosatetraenoate. Interestingly, Reddy *et al.* (22) have reported the detection of 9,15-diHETE along with other diHETE from the oxidation of arachidonic acid by potato tuber lipoxygenase.

In this work, we have demonstrated that *P. latissima* produces oxylipins quite different from those found previously in other red marine algae. All of these reasonably derive from a 9(*S*)-lipoxygenase-initiated metabolic pathway, in this case acting on arachidonic acid to produce 9(*S*)-hydroperoxy-eicosatetraenoic acid (9(*S*)-HpETE) as a central intermediate (Scheme 1). Simple peroxidase-type reduction of this hy-

droperoxide would yield 9(*S*)-HETE (**1**). Rearrangement of this 9(*S*)-HpETE, possibly catalyzed by a hydroperoxide dehydrase or hydroperoxide isomerase (23), could yield the hepoxilin-like metabolites **5** and **7**. Alternatively, through an epoxyallylic carbocation intermediate (24) with resulting proton losses and rearrangements as for colneleic acid (15), 9(*S*)-HpETE could be converted to polyneuric acid (**3**). Finally, 9,15-diHETE (**9**) could be produced by further lipoxygenase-type oxygenation and reduction of 9(*S*)-HpETE or 9(*S*)-HETE (22).

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Photo-Initiated Peroxidation of Lipids in Micelles by Azaaromatics

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ABSTRACT: The monoazaaromatics, pyridine (**1**), hexyl nicotinate (**2**), and quinoline (**3**) and diazaaromatics, pyrimidine (**4**) and purine (**5**), readily act as photo-initiators for the peroxidation of methyl linoleate in 0.50 M SDS at 37°C giving free radical chain oxidations of linoleate. Quantitative kinetic runs on the order in substrate, RH, and in the rate of chain initiation, R_i, showed that the classical rate law for autoxidation, $-d[O_2]/dt = (k_p/(2\ k_t^{1/2}))[RH] \times R_i^{1/2}$, is applicable to these photo-initiated oxidations. The oxidizability of methyl linoleate under these conditions is 2.92×10^{-2} $M^{-1/2}$ $s^{-1/2}$. These peroxidations were inhibited by chromanol phenolic antioxidants of the vitamin E class, such as lipid-soluble 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMHC) and water-soluble 2-carboxy-2,5,7,8-tetramethyl-6-hydroxychroman (Trolox) and derived rate constants for inhibition of peroxidation were k_{inh} (PMHC) = 4.35×10^4 M^{-1} s^{-1} and k_{inh} (Trolox) = 2.81×10^4 M^{-1} s^{-1} during inhibited oxidation of methyl linoleate photo-initiated by **4**. The products from photo-initiated peroxidation of methyl linoleate by **1** through **5** were determined by reduction and high-performance liquid chromatography analyses to be the 9- and 13-positional hydroperoxides of the four geometrical isomers: *cis*-9, *trans*-11 (**6**), *trans*-10, *cis*-12 (**7**), *trans*-9, *trans*-11 (**8**), and *trans*-10, *trans*-12 (**9**)–octadecadienoates typical of the free radical chain mechanism of lipid peroxidation. Products from dye-sensitized oxidation by Methylene Blue or Rose Bengal of methyl linoleate gave a product distribution of six hydroperoxides typical of oxidation by singlet oxygen. Thermal or photo-initiated peroxidation of methyl linoleate in SDS gave some selectivity of oxidation at the 13-position of the linoleate chain. The ratio of 13- to 9-oxidation varied in the range 1.23 to 1.14 as the *cis/trans* to *trans/trans* ratio of geometrical isomers varied from 0.44 to 1.25 during photooxidation of increased amounts of linoleate in SDS. This selectivity is attributed to loss of the *pseudo* symmetry around the pentadienyl system in the lipid chain in the SDS system during the peroxidation.

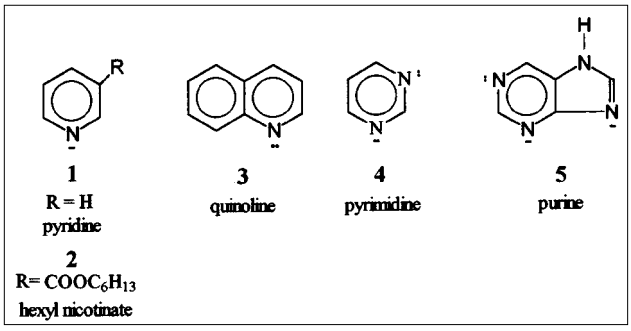
Lipids 32, 237–245 (1997).

Six-membered nitrogen heterocyclic aromatic compounds, azaaromatics and their derivatives, are widely distributed in nature. Their photochemistry has received considerable attention, but their possible role as initiators of photooxidation, the purpose of this investigation, is relatively unknown. This is surprising since the photogenerated (n,π^*) states of various azaaromatics are well known to abstract H-atoms from donors. Such studies have included a variety of monoazaaromatics (1–9), such as pyridine, quinoline, isoquinoline and acridine, and a number of diazaaromatics (10,11). The earlier research on the photochemistry of azaaromatics has been reviewed (12).

Since hydrogen abstraction from organic substrates by n,π^* states of various azaaromatics has been well established, this process is expected to be a method of photo-initiated peroxidation of organic substrates. The role that biologically important azaaromatics, such as the DNA/RNA bases purine and pyrimidine, might play in photooxidation is of particular interest because of the role this process could play in initiating degenerative diseases such as skin cancer.

Although the advantages of the photochemical method of lipid peroxidation have recently been reviewed (13), there was no example cited where an azaaromatic was used as the photo-initiator. The objective of the research reported herein was to employ these compounds in a quantitative study of lipid peroxidation in a biphasic system to mimic a membrane. Consequently we now report on the following quantitative study of photo-initiated lipid peroxidation.

(i) The aromatic azaaromatic amines selected include monoazaaromatics (Scheme 1): pyridine (**1**), hexyl nicotinate (**2**), and quinoline (**3**) and the model bases pyrimidine (**4**) and purine (**5**).



SCHEME 1

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Abbreviation: PMHC, pentamethyl-6-hydroxychroman.

(ii) The system selected for the reactions is 0.50 M SDS. This biphasic aqueous/hydrophobic system has proved useful as a model system, which mimics lipid membranes in some respects and is convenient for quantitative kinetic studies, such as kinetic orders of oxygen absorption, and analytical studies (14–24).

(iii) The substrate used is linoleate (methyl linoleate and linoleic acid). Product studies performed on the peroxidized substrate provide important evidence on the mechanism of peroxidation, e.g., free radical chain peroxidation as compared to reaction products resulting from attack by singlet oxygen. Singlet oxygen reactions are also studied in the SDS system by sensitization in the presence of Methylene Blue or Rose Bengal.

MATERIALS AND METHODS

Materials. Methyl linoleate and linoleic acid were obtained from Nu-Chek-Prep (Elysian, MN) (purity >99%) and stored in sealed vials at –30°C until used. Trolox was purchased from Aldrich Chemicals (Milwaukee, WI). 2,2,5,7,8-Pentamethyl-6-hydroxychroman (PMHC) was synthesized by a known procedure (25). Pyridine, hexyl nicotinate, quinoline, pyrimidine, and purine were obtained from Aldrich Chemicals. Quinoline was distilled under reduced pressure before use. Benzophenone was purchased from Fisher Scientific and recrystallized from pentane before use. Methylene Blue and Rose Bengal were obtained from Aldrich Chemicals. SDS was electrophoresis purity obtained from Bio-Rad (Richmond, CA). The phosphate buffer (pH 7.0) was prepared from 0.05 M each of Na_2HPO_4 and NaH_2PO_4 in deionized distilled water containing 10^{-4} M EDTA. The buffer was passed through a column of Bio-Rad Chelex 100 (50–100 mesh) to remove traces of heavy metal ions. Organic solvents used were Omni Solv (BDH, Toronto, Ontario, Canada) spectroscopic grade.

Preparations. Solutions of 0.50 M SDS were prepared in phosphate buffer and stored at 0°C in the dark until used. Solutions of the various photo-initiators and of the phenolic antioxidant, PMHC, were prepared in the 0.50 M SDS by vortex stirring known amounts of each in known volumes of SDS. Their concentrations were checked by diluting aliquots with methanol and water and by comparison of their ultraviolet spectra with that of solutions of known concentration prepared separately in methanol and water. The initiator-SDS stock solutions were stored at 0°C and in the dark before use.

The autoxidation/inhibition procedures. Autoxidations were generally carried out at 37°C/760 torr under oxygen using a sensitive, calibrated dual channel pressure transducer system that is described in detail elsewhere (26). Autoxidations using pyridine as the photo-initiator were carried out using a YSI Model S-18053 Oxygen Monitor System with a Clark oxygen electrode (Yellow Springs, OH).

In a typical experiment, 2.00 mL samples of 0.50 M SDS–phosphate buffer were equilibrated at 37°C under oxygen in the pressure transducer system. Then a known amount of azaaromatic solubilized in a few μL of 0.50 M SDS was injected into the sample channel cell followed by a known amount of linoleic acid or methyl linoleate. The sample was allowed to equilibrate again by shaking the system under oxygen. When a stable baseline was observed, the sample was irradiated with a 200 W super pressure Hg arc lamp through a glass filter. The lamp intensity was monitored throughout the experiment using a fiber optics cable attached to a phototube and picoammeter detector. Kinetic measurements with respect to substrate were performed by adding the substrate from a μL precision syringe and measuring the oxygen uptake after each addition. The effect of variation of light intensity was followed by using a series of seven neutral density filters of known transmittance. Inhibition periods by PMHC or Trolox were obtained by injecting known amounts of the inhibitors and measuring the time (τ) until the oxygen uptake rate returned to the uninhibited rate.

Analyses. Concentrations of the azaaromatics and of PMHC prepared in the stock 0.50 M SDS solutions were checked by diluting known aliquots in (1:1) methanol/distilled water. The spectra were then recorded on a Shimadzu UV-1201 Spectrometer (Kyoto, Japan). Trolox was prepared in a known concentration in pH 7.0 phosphate buffer. Analyses for oxidation products were carried out as reported earlier (27). Samples from an oxidation run were immediately reduced to the hydroxy derivatives by triphenylphosphine using a known procedure (18). The products were extracted in hexane and analyzed at 234 nm on a Hewlett-Packard HP1050 HPLC (Palo Alto, CA) interfaced for computer analyses. Separations were achieved using a LiChrosorb Si60 (E. Merck, Darmstadt, Germany) (5 μm) silica column and a solvent system containing hexane/isopropyl alcohol/acetone mixture in the ratio 992:4:4. The solvent system was distilled before use. Gas–liquid chromatography analyses were done on a Varian Model 3700 gas chromatograph (Palo Alto, CA). The concentrations of quinoline used in a run (initial and final) were measured from aliquots containing *n*-butanol as internal standard. The analyses were performed on a $1' \times ¼''$ 8% Carbowax column (Chromatographic Specialities, Brockville, Ontario, Canada) using helium at 30 mL/min and 130°C for 3 min, followed by a program at 20°C/min to 220°C. The concentrations of hexyl nicotinate and of pyrimidine used in oxidations were determined by extraction of the samples with hexane and gas–liquid chromatography analysis on an $8' \times ¼''$ 10% Silar column (Chromatographic Specialities) using methyl benzoate as an internal standard. Helium was the carrier gas at 30 mL/min and 120°C for 1 min, followed by a program at 15°C/min to 210°C.

RESULTS

The quantitative kinetic method. Free radical chain autoxidations of organic substrates are well known to follow a classical rate law for oxygen uptake, Equation 1:

$$\frac{-d[O_2]}{dt} = \frac{k_p}{(2k_t)^{1/2}} \times [RH] \times R_i^{1/2} \qquad [1]$$

where k_p is the rate constant for chain propagation by hydrogen abstraction from the substrate, RH, by peroxyl radicals and 2 k_t is the rate constant for termination by recombination of peroxyl radicals. This classical equation applies to the autoxidation of many organic substrates in homogeneous solution, and it is also known to be applicable in certain cases to substrates in biphasic systems such as aqueous micelles and membranes (17,20,24). When this relationship applies, one can quantify the susceptibility of a substrate to undergo peroxidation, known as its oxidizability, by Equation 2.

$$\text{Oxidizability} = \frac{k_p}{(2k_t)^{1/2}} = \frac{-d[O_2]/dt}{[RH] \times R_i^{1/2}} \quad [2]$$

For quantitative studies, the rate of chain initiation, R_i, must be known and controlled. This is often done by using thermal azo initiators with known rate constants of decomposition and efficiency. In our present photochemical method, it is accomplished by using a monitored light source, selected filters, and known amounts of the azaaromatics. The R_i in each experiment was measured by adding a phenolic antioxidant (ArOH), known to trap two peroxyl radicals (e.g., with a stoichiometric factor, n, of 2), and by measuring the inhibition period, τ , during which oxygen uptake is suppressed. Under these controlled conditions, Equation 3 applies.

$$R_i = n[\text{ArOH}]/\tau \quad [3]$$

During inhibited peroxidation, peroxyl radicals are terminated by mechanisms represented by Equations 4 and 5.

$$\text{ROO}^{\cdot} + \text{ArOH} \xrightarrow{k_{inh}} \text{ROOH} + \text{ArO}^{\cdot} \quad [4]$$

$$\text{ROO}^{\cdot} + \text{ArO}^{\cdot} \xrightarrow{\text{fast}} \text{nonradical products} \quad [5]$$

Under these conditions the suppressed oxygen uptake is given by Equation 6.

$$\frac{-d[O_2]}{dt} = \frac{k_p}{k_{inh}} \times [RH] \times \frac{R_i}{n[\text{ArOH}]} \quad [6]$$

An integrated version of this equation, Equation 7, is useful to measure the antioxidant activities of inhibitors.

$$\Delta[O_2]_t = \frac{-k_p}{k_{inh}} \times [RH] \times \ln\left(1 - \frac{t}{\tau}\right) \quad [7]$$

The absolute rate constant for inhibition, k_{inh} , is determined from a linear plot of the oxygen uptake during the course of the inhibition period, $\Delta[O_2]$, vs. $-\ln(1 - t/\tau)$ where the slope $= (k_p/k_{inh})[RH]$.

These quantitative methods proved useful for kinetic measurements, e.g., of k_{inh} values, in SDS employing thermal azo initiators (21) and earlier using photochemical initiation by benzophenone (18). In the present study, it was desirable first to determine if the azaaromatics **1–5** photo-initiate peroxidation of linoleate in SDS micelles and then determine the kinetic orders of oxygen uptake in substrate (linoleate) and in the rate of chain initiation, R_i, for the various azaaromatic photo-initiators used; in other words determine if the classical rate law, Equation 1, is applicable for this method of initiation in this biphasic system.

Kinetic orders in substrate and R_i. The azaaromatics used exhibited characteristic absorption in the near ultraviolet in methanol/water (1:1) as follows: pyridine (**1**), λ_{max} 250.5 nm, ε = 2617; hexyl nicotinate (**2**), λ_{max} 243, ε = 3072; quinoline (**3**), λ_{max} 313, ε = 2818; pyrimidine (**4**), λ_{max} 291, ε = 388 and purine (**5**), λ_{max} 262, ε = 7943. Measurable oxygen uptake occurred on irradiation of a 0.50 M SDS solution of linoleic acid or methyl linoleate containing each of these amines. Although the ultraviolet maxima for compounds **1**, **2**, and **5** are well below the "cut-off" for the glass filter used, their very broad absorption bands tail off past the cut-off allowing sufficient absorption of light to initiate photooxidation of the reactive substrate.

In order to test the effect of varying the micellar linoleate concentration on the oxygen uptake, a series of experiments were conducted with each amine initiator. Linoleate concentrations were varied over at least a fivefold range. The results of these kinetic measurements involving the monoazaaromatics are illustrated in Figure 1 (concentrations given assume that the linoleate and initiators partition into the SDS phase). Linear plots in linoleate concentration (not shown) were also observed using the diazaaromatics **4** (0.0197 M) and **5** (0.0272 M) and linoleate concentrations ranges of 0.0850 to 0.483 and 0.0794 to 0.435 M. Quinoline (2.19 mM) initiator in homogeneous solution of methyl linoleate (0.0578 to 0.167 M) in *tert*-butyl alcohol gave a kinetic order of 1.08 in substrate (not shown). Linear plots for the kinetic order in micellar linoleate were observed in all cases for the concentrations ranges studied and the kinetic orders in oxygen uptake were close to unity; all lying in the range 0.96 to 1.12.

The dependence of the rate of oxygen consumption on variation of the rate of initiation (R_i) was determined by vary-

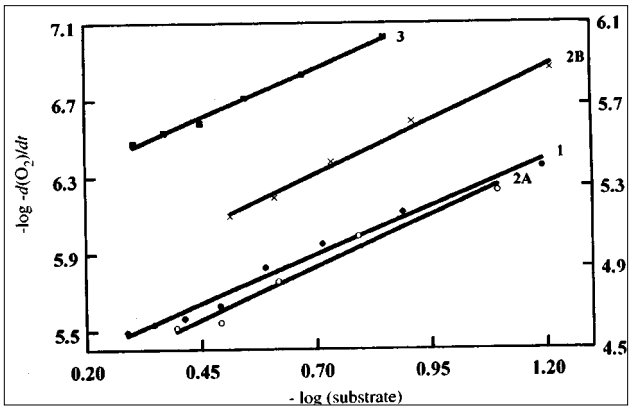

FIG. 1. Kinetic order plots of oxidation with respect to linoleate concentration for photo-initiated peroxidation by monoazaaromatics in 0.50 M SDS, phosphate buffer, pH 7.0, at 37°C. 1, linoleic acid (0.063 to 0.515 M), pyridine (0.618 M). 2A, methyl linoleate (0.0805 to 0.402 M), hexyl nicotinate (0.0186 M). 2B, linoleic acid (0.0613 to 0.306 M), hexyl nicotinate (0.0187 M). 3, methyl linoleate (0.0707 to 0.495 M), quinoline (0.0217). The y range for line 3 is from 4.5 to 6.0.

ing the light intensity as reported before by initiation using benzophenone (18). Typical results from at least five different light intensities are shown in Figure 2 for the monoazaaromatic initiators. Similar kinetic orders in light intensity (not shown) were obtained using the diazaaromatics **4** (0.0197 M) with methyl linoleate (0.402 M) and **5** (0.0272 M) and methyl linoleate (0.4345 M). Experiments to measure the dependence of the rate of oxidation on light intensity in solution of *tert*-butyl alcohol (not shown) using methyl linoleate (0.0759 M) and quinoline (2.19 mM) gave a kinetic order of 0.49. The results show linear variation of the rate of oxidation with light intensity across a wide range of micellar linoleate concentrations (0.0707 to 0.4345 M), and the reaction orders obtained from the slopes were approximately half order, all lying in the range of 0.42 to 0.60.

Kinetic results from inhibited peroxidation. Typical experimental profiles of oxygen uptake of uninhibited and inhibited peroxidation of linoleate in 0.50 M SDS are shown in Figures 3–5. These are typical results for photo-initiation using various combinations of water-soluble initiators (pyridine **1**, pyrimidine **4**, and purine **5**) and lipid-soluble ones (hexyl nicotinate **2** and quinoline **3**) and inhibition by the water-soluble antioxidant, 2,5,7,8-tetramethyl-2-carboxy-6-hydroxychroman (Trolox), and by the lipid-soluble one, 2,2,5,7,8-PMHC, both known to trap two peroxyl radicals in chain terminating peroxidation (28,29) (Equation 3, $n = 2$). For example, Figure 3 shows the inhibiting effect of PMHC for reaction initiated by water-soluble **1**, and inhibition by Trolox during initiation by the lipid-soluble derivative, **2**, and Figure 4 shows results from the combination of initiation by lipid-soluble quinoline and inhibition by Trolox. Figure 5 demonstrates that reactions initiated by water-soluble pyrimidine or purine can be inhibited effectively by either Trolox or PMHC.

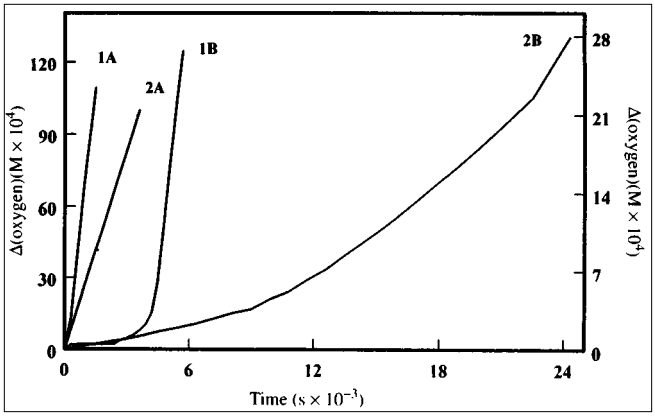

FIG. 3. Peroxidation of methyl linoleate photo-initiated in 0.50 M SDS, pH 7.0, at 37°C. 1A, uninhibited reaction. Methyl linoleate (0.097 M), pyridine (0.182 M). 1B, inhibited by PMHC (1.124×10^{-8} mol). The scale of oxygen uptake is shown on the right for 1A and 1B as 0 to 28 $M \times 10^{-4}$. 2A, uninhibited reaction. Methyl linoleate (0.241 M), hexyl nicotinate (0.0187 M). 2B, inhibited by Trolox (0.652×10^{-8} mol).

(i) Oxidizability. The results on kinetic orders in substrate and light intensity indicate that the classical rate law is applicable to peroxidation of linoleate in micelles under the conditions used. In addition, the R_i can now be determined from the results of these inhibition studies and calculated using Equation 3. Consequently, requirements are met to determine the susceptibility of linoleate to undergo free radical peroxidation, its oxidizability, by application of Equation 2. Results of such measurements using methyl linoleate in 0.50 M SDS employing the azaaromatic photo-initiators and PMHC or Trolox to measure the R_i are summarized in Table 1. The results show the oxidizability, $k_p/(2\,k_t)^{1/2} = 2.92 \pm 0.29 \times 10^{-2}$ $M^{-1/2}\,s^{-1/2}$, to be the same, within experimental error, for the various methods of initiation and inhibition. Substantial kinetic chain lengths, in the range of 77 to 253 for the uninhibited reaction, were obtained under the conditions used.

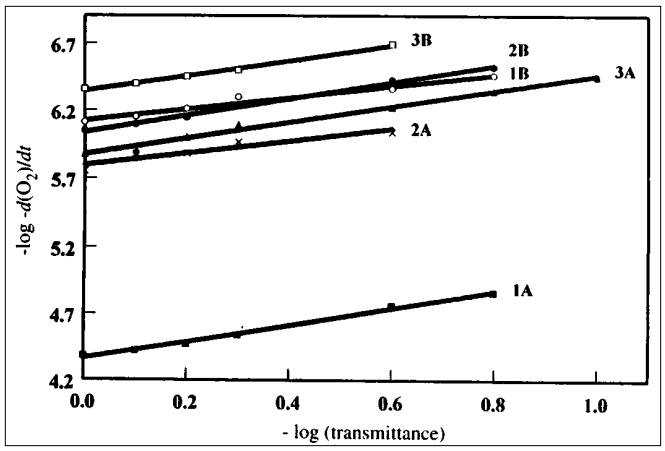

FIG. 2. Kinetic order plots of oxidation with respect to light intensity (transmittance) for photo-initiated peroxidation of linoleate in 0.50 M SDS, phosphate buffer, pH 7.0, at 37°C. 1A, methyl linoleate (0.0966 M), pyridine (0.306 M). 1B, linoleic acid (0.129 M), pyridine (0.618 M). 2A, methyl linoleate (0.241 M), hexyl nicotinate (0.0186 M). 2B, linoleic acid (0.306 M), hexyl nicotinate (0.0187 M). 3A, methyl linoleate (0.0707 M), quinoline (0.0217 M). 3B, linoleic acid (0.130 M), quinoline (0.0252 M).

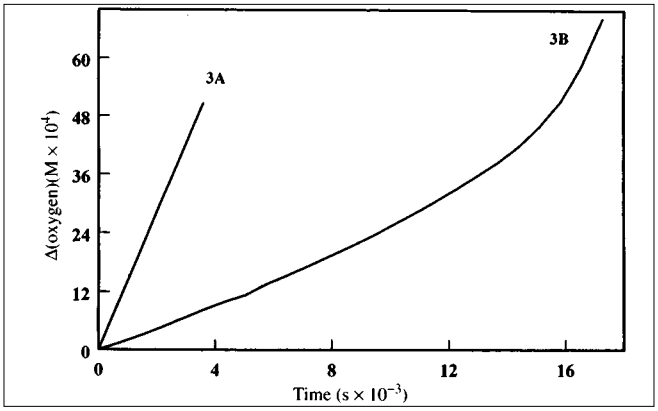

FIG. 4. Peroxidation of methyl linoleate (0.566 M) in 0.50 M SDS, pH 7.0, at 37°C, photo-initiated by quinoline (0.0192 M). 3A, uninhibited reaction. 3B, inhibited by Trolox (4.10×10^{-8} mol).

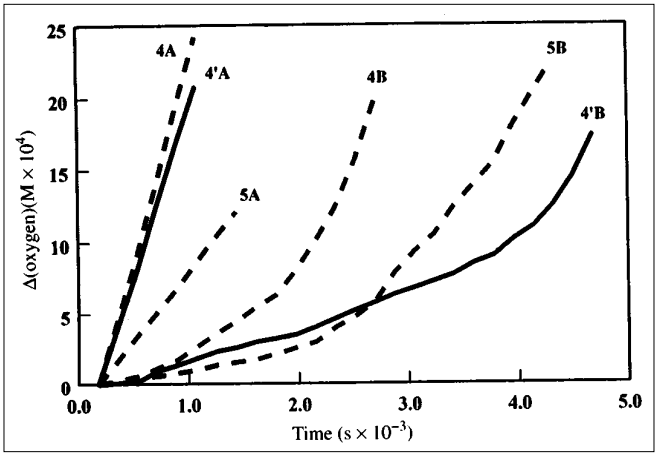

FIG. 5. Peroxidation of methyl linoleate photo-initiated in 0.50 M SDS, pH 7.0, at 37°C. The same light intensities were used for each pair of uninhibited and inhibited experiments. 4A, uninhibited reaction, methyl linoleate (0.563 M), initiated by pyrimidine (0.284 M). 4B, inhibited by Trolox (0.639×10^{-8} mol). 4′A, uninhibited reaction, methyl linoleate (0.503 M), initiated by pyrimidine (0.284 M). 4′B, inhibited by PMHC (1.56×10^{-8} mol). 5A, uninhibited reaction, methyl linoleate (0.435 M), initiated by purine (0.0349 M). 5B, inhibited by Trolox (0.205×10^{-8} mol).

(ii) Antioxidant activities. In order for kinetic studies to be applied to the determination of activities of antioxidants during peroxidation, specific conditions must be met, namely, (i) there must be a definite inhibition period during which oxygen uptake is suppressed so that this period (τ) can be measured, (ii) there should be a measurable chain reaction remaining during this inhibition period so that the steady-state approximation (Equation 6) is applicable and suppressed oxygen uptake can be measured, and (iii) the propagation rate constant, k_p, must be known. Linear plots of $\Delta[O_2]$ vs. $-\ln(1 - t/\tau)$ (Equation 7) and the known k_p for linoleate in SDS (15) were used to obtain the rate constant of inhibition for lipid-soluble PMHC ($k_{inh} = 3.42 \times 10^4$ M^{-1} s^{-1}) and for water-soluble Trolox ($k_{inh} = 4.35 \times 10^4$ M^{-1} s^{-1}) during photo-initiation by water-soluble pyrimidine.

Analyses of lipid hydroperoxides and of initiators. It is well known that the free radical peroxidation of methyl linoleate produces four conjugated regio-geometric isomers: the 9- and 13-perhydroxy *cis, trans* (*c,t*) and *trans,trans* (*t,t*) isomers (30,31). In contrast, singlet oxygen oxidation of methyl linoleate yields both conjugated and nonconjugated isomers in approximately equal amounts (32,33), presumably by the concerted ene reaction with singlet oxygen, rather than *via* a peroxyl radical intermediate.

We carried out product studies of the isomers formed by photo-initiated oxidation of methyl linoleate by different initiators. The results of these studies are given in Table 2. In addition, the trend in product formation with linoleate concentration was followed during photo-initiation by pyrimidine, as shown. In each case the isomers were separated and analyzed as the corresponding four isomeric alcohols (see Fig. 6), formed by reduction of the products with triphenylphosphine after low extent of oxidation (less than 5%). In all cases, the four isomers formed (**6–9**) are consistent with a free radical peroxidation pathway.

In addition, a few experiments were conducted using dye-sensitized oxidation of methyl linoleate in 0.50 M SDS, conditions which lead to reaction by singlet oxygen and an entirely different profile of products. Under these conditions, six isomers were formed with both conjugated and nonconjugated structures. These results are outlined in Table 3.

In the system photo-initiated by pyrimidine, the trend in the ratio of *c,t* to *t,t* isomers with different linoleate concentration was followed. The data for these results are included in Table 2. A plot of the trend is illustrated in Figure 7, along with the trend in oxidation products formed at position 13 compared to position 9 at the different concentrations (the 13:9 ratio). The linear variation in *c,t* to *t,t* ratio with linoleate concentration follows the expected trend. As Porter (34) reported, the ratio of *c,t*/*t,t* isomers depends directly on the H-atom donating ability of the system, here the concentration of the bisallylic system, linoleate. However, our observed variation of the 13:9 ratio is unusual, since this ratio was reported to be 1:1 in all other cases, even when experiments were conducted which should reveal if there is any preference for oxidative attack at the 9- or 13-position of the chain (27,35). We also found that the 13:9 ratio varies from unity

TABLE 1
Kinetics of Photooxidation of Methyl Linoleate with Azaaromatic Compounds in 0.50 M SDS (pH 7.0)

RH (M)	Photo-initiator (mol × 10^4)	$-d[O_2]/dt$ (M/s × 10^6)[a]	Inhibitor (nmol)	τ (s × 10^{-3})	R_i^b (M/s × 10^8)	ν[c]	$\frac{k_p^d}{(2\ k_t)^{1/2}}$ ($M^{-1/2}$ $s^{-1/2}$ × 10^2)
0.105	Pyridine (5.46)	3.53	PMHC (12.4)	4.29	1.38	256	2.84
0.253	Hexyl nicotinate (0.05)	0.66	Trolox (5.22)	19.03	0.22	301	2.55
0.563	Pyrimidine (5.68)	2.73	Trolox (6.39)	2.26	2.50	109	3.06
0.503	Pyrimidine (5.68)	2.29	PMHC (15.6)	4.17	3.00	76	2.64
0.507	Purine (0.992)	1.00	PMHC (2.88)	5.40	0.43	233	3.03
0.435	Purine (0.698)	1.19	Trolox (2.05)	2.54	0.65	183	3.41

[a]Rate of oxygen consumption for uninhibited reaction.
[b]The rate of chain initiation, determined using the relationship, $R_i = 2[ArOH]/\tau$, where τ = the induction period.
[c]Kinetic chain length of the uninhibited reaction, $\nu = (-d[O_2]/dt)/dt$.
[d]The oxidizability of methyl linoleate calculated using Equation 2; PMHC, pentamethyl-6-hydroxychroman.

TABLE 2
Product Distributions Obtained from Product Studies Involving Reactions Photo-Initiated by Azaaromatic Compounds and Benzophenone of Methyl Linoleate (ML) or Linoleic Acid (LA) in 0.50 M SDS (pH 7.0)

Photo-initiator (μmol)	Substrate (M)[a]	**6**[b]	**7**	**8**	**9**	$\frac{c,t}{t,t}$	$\frac{\mathbf{13}}{\mathbf{9}}$
Benzophenone (2.94)	ML (0.161)	0.13	0.11	0.41	0.35	0.32 ± 0.01	1.15 ± 0.03
Pyridine (618.2)	LA (0.257)	0.23	0.18	0.33	0.25	0.69, 0.74	1.27, 1.31
Hexyl nicotinate (4.92)	ML (0.161)	0.16	0.12	0.40	0.32	0.40 ± 0.06	1.24 ± 0.04
Quinoline (4.92)	ML (0.566)	0.29	0.18	0.28	0.25	0.96 ± 0.04	1.44 ± 0.09
Purine (99.2)	ML (0.507)	0.26	0.22	0.29	0.23	0.90 ± 0.02	1.23 ± 0.06
Pyrimidine (1544.3)	ML (0.085)	0.17	0.14	0.38	0.31	0.44 ± 0.02	1.23 ± 0.07
Pyrimidine (567.1)	ML (0.169)	0.21	0.16	0.34	0.29	0.59 ± 0.01	1.22 ± 0.03
Pyrimidine (567.1)	ML (0.253)	0.22	0.18	0.35	0.25	0.61 ± 0.06	1.18 ± 0.07
Pyrimidine (1544.3)	ML (0.402)	0.28	0.24	0.27	0.23	1.04 ± 0.02	1.17 ± 0.01
Pyrimidine (567.1)	ML (0.507)	0.29	0.26	0.24	0.21	1.25 ± 0.01	1.14 ± 0.06

[a]A micellar reaction volume of 2.5×10^{-4} L estimated for 2.00 mL of 0.50 M SDS was used in concentration calculations (Ref. 19).
[b]The structures of the isomers **6** to **9** are given in Figure 6.

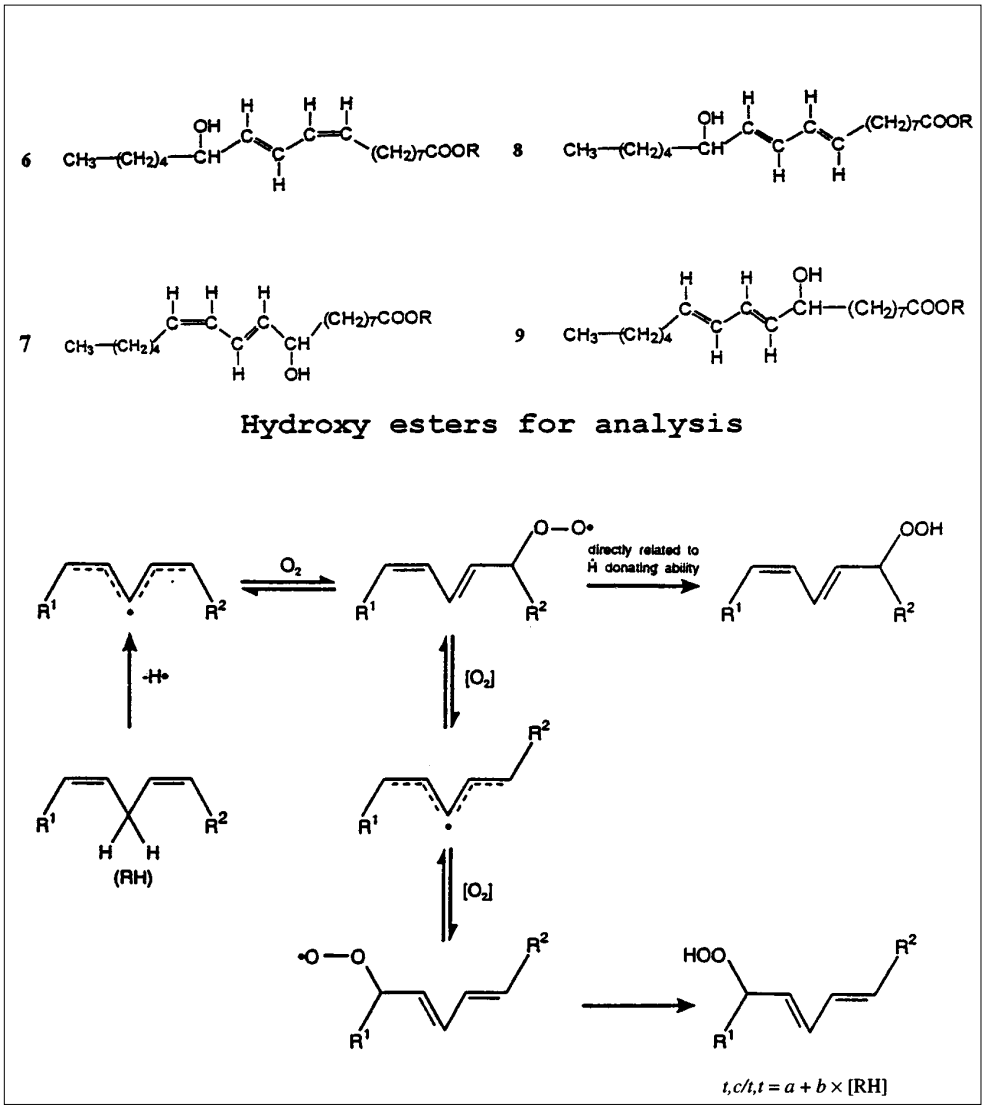

FIG. 6. The 9- and 13-hydroxy *cis,trans* and *trans,trans* hydroxy esters derived from peroxidized linoleate, and a scheme for formation of the hydroperoxides.

TABLE 3
Data from Product Studies Involving Singlet Oxygen-Initiated Photooxidation Reactions of Methyl Linoleate in 0.50 M SDS (pH 7.0)

Photo-sensitizer (μmol)	RH (M)	**6**	**7**	**8**	**9**	**10**[a]	**11**[a]	$\frac{c,t}{t,t}$	$\frac{\mathbf{13}}{\mathbf{9}}$
Methylene Blue (1.324)	0.483	0.47	0.43	0.04	0.04	0.01	0.01	10.03 ± 1.01	1.11 ± 0.04
Rose Bengal (0.132)	0.483	0.43	0.40	0.07	0.08	0.01	0.01	5.68 ± 0.07	1.01 ± 0.01

[a]The nonconjugated 10-hydroxy *trans*-8, *cis*-12 and 12-hydroxy *cis*-9, *trans*-13-octadecadienoate products (see Ref. 32). The ratio of **10** to **11** was unity, but these ratios are not on the same scale as isomers **6** to **9** because of the different absorptivities of these isomers at the wavelength used for high-performance liquid chromatography analyses.

when oxidation was initiated with a thermal azo initiator (di-*tert*-butyl hyponitrite) in SDS, and this trend is also shown in Figure 7.

Analyses for loss of initiators during the photolyses were performed by ultraviolet spectrophotometry and gas–liquid chromatography. Pyridine is very soluble in the aqueous phase, and we were not successful in extracting it in quantitative yield from the aqueous phase with an organic solvent. In addition, it appears that a significant amount remains in the vapor state above the sample. This factor also caused experimental difficulty in determining the order of oxygen consumption in light intensity using the pressure transducer system. That procedure assumes that the pyridine remains entirely in the aqueous–micellar phase. For this reason, the kinetic order was determined using the oxygen electrode where the sensor is isolated from the vapor phase.

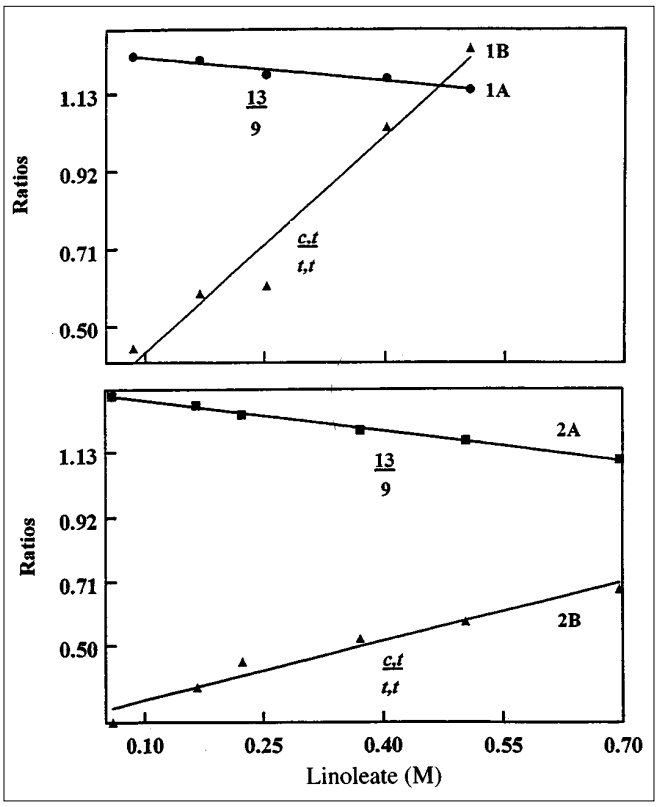

FIG. 7. Concentration dependence in linoleate of product ratios of *cis,trans* to *trans,trans* hydroxy fatty esters [(**6** + **7**)/(**8** + **9**), Fig. 6] and 13:9 ratios of position isomers from peroxidation of linoleate in 0.50 M SDS. 1A, Ratio of 13- and 9-hydroperoxides formed from oxidation of methyl linoleate, photo-initiated by pyrimidine. Data taken from Table 2. 1B, Ratio of *cis,trans* and *trans,trans* hydroperoxides formed from oxidation of methyl linoleate, photo-initiated by pyrimidine. Data taken from Table 2. 2A, Ratio of 13- and 9-hydroperoxides formed from oxidation of linoleic acid, thermally initiated by di-*tert*-butylhyponitrite. Data taken from Reference 18. 2B, Ratio of *cis,trans* and *trans,trans* hydroperoxides formed from oxidation linoleic acid, thermally initiated by di-*tert*-butylhyponitrite. Data taken from Reference 18.

Experiments using hexyl nicotinate as the photo-initiator showed that the maximum amount lost during the course of an experiment was 5%, according to analysis by ultraviolet spectrophotometry of an experimental sample from which the initiator was extracted in a layer of hexane. Gas chromatography analysis of a sample extracted using the same procedure indicated a loss of only 2% of the hexyl nicotinate.

Ultraviolet analyses were performed directly on the experimental samples which were photo-initiated by pyrimidine and purine. These samples contained the photo-initiator, SDS, and methyl linoleate, and analyses were performed prior to, and following, the photooxidation reactions. The analyses indicated that there was no loss of pyrimidine, and that the maximum amount of purine lost even after extensive irradiation was only 7%.

DISCUSSION

Our results demonstrate for the first time that both water-soluble and lipid-soluble azaaromatics readily photo-initiate peroxidation by near ultraviolet irradiation of an unsaturated lipid in a model biphasic system of SDS micelles. All of the data indicate a typical free radical chain peroxidation. Appreciable kinetic chain lengths were observed for the oxidations and the kinetic orders in substrate (linoleate) concentration and R_i show that the oxidations follow the classical rate law for autoxidation. The oxidizability of methyl linoleate was determined to be 2.92×10^{-2} $M^{-1/2}$ $s^{-1/2}$, similar to the value reported for linoleic acid in SDS using a thermal initiator (4.48×10^{-2} $M^{-1/2}$ $s^{-1/2}$) (19). Unlike the case for thermal initiation, there is not a simple relationship between the oxygen uptake and the azaaromatic employed in this study (see Table 1). The efficiency of initiation using these photoinitiators will depend on the light absorbed which in turn varies with the molar absorptivities. In addition the system is complicated by different distributions of the initiators between the aqueous phase and reaction phase containing the substrate in the micelles and these distributions are not known.

These peroxidations were readily inhibited by a water-soluble or lipid-soluble antioxidant of the hydroxychroman (vitamin E) class. Typical antioxidant activities for the antioxidants used, lipid-soluble PMHC, $k_{inh} = 3.42 \times 10^4$ M^{-1} s^{-1} and water-soluble Trolox, $k_{inh} = 4.35 \times 10^4$ M^{-1} s^{-1}, are in agreement with those found in SDS micelles for PMHC by thermal methods of initiation ((21), $k_{inh} = 3.96 \times 10^4$ M^{-1} s^{-1}) and for Trolox by photo-initiation methods ((18), $k_{inh} = 2.81 \times 10^4$ M^{-1} s^{-1} at 30°C). Oxidation was not initiated in the absence of the azaaromatics on methyl linoleate in SDS micelles under our conditions.

The results from high-performance liquid chromatography analyses of the hydroperoxides formed during a photo-initiated run involving azaaromatics are consistent with a reaction mechanism involving H-abstraction from the lipid doubly allylic $-CH_2-$ group followed by attack by oxygen at the 9- and 13-positions of the resulting pentadienyl radical (Fig. 6). There was no evidence for oxidation by singlet oxygen, which might be expected to form by energy transfer, since we have confirmed that this pathway gives rise to different products from linoleate during dye-sensitized peroxidation in SDS.

The observation of 13:9 ratios higher than unity (Table 2) is of interest and appears to be unique. We also find an inverse linear trend of the 13:9 ratios when plotted along with the *cis,trans/trans,trans* ratios, with higher 13:9 ratios corresponding to lower *c,t/t,t* ratios for both photo-initiated oxidation and thermal oxidation using the earlier procedure (18; Fig. 7). We speculate that the unusual 13:9 ratios are due to several factors. (**1**). The *cisoid* and *transoid* conformers of the pentadienyl radical are not in thermal equilibrium (36,37), and peroxidation at the *transoid* end of the linoleate pentadienyl radical is more facile than at the *cisoid* terminus (38). (**2**). The ratios of the kinetic products to thermodynamic products (*c,t* to *t,t*) are directly related to the H-atom donating ability of the medium (34). (**3**). The linoleate chain is expected to be in a nonuniform environment in our SDS system. Bilayer soap molecules, such as palmitate, are known to exhibit increased conformational freedom toward the chain terminus (39) and SDS above the critical micelle concentration is known to undergo a phase change from micellar to such bilayer structures (Robinson, B.H., Private Communication). So the linoleate chain in 0.50 M SDS is expected to be subject to nonuniform effects. If the polar headgroup is positioned near the SDS headgroup as is expected, the hydrocarbon tail would be in the region of lower microviscosity where the derived peroxyl radical initially formed at position 13 would be free to rotate to the *transoid* conformation. On the other hand, the larger $C(9)HOO(CH_2)_7COOCH_3$ at position 9 would be more constrained from rotation about the C(9) to C(10) bond axis. As a result, position 13 more readily sets up the *transoid* conformer for more rapid oxodation. This possibility is shown schematically in Figure 8.

Analyses for the azaaromatic initiators indicate that they are recovered almost completely unreacted at the end of the usual photooxidation. This recycling of the initiators is a useful phenomenon because it provides for a constant R_i throughout the experiments. A possible scheme for this recycling is given in Figure 9 for for compound **2** whereby it is recovered by H-atom transfer from the excited pyridyl radical to oxygen and/or to a lipid peroxyl radical. A similar pathway was proposed earlier for the recycling of benzophenone used in photooxidation (18).

The SDS biphasic system is a useful one to use for these initial studies employing the azaaromatics as photo-initiators. Studies are in progress to apply these methods to the photoperoxidation of phospholipid membranes where the results are expected to more closely mimic the behavior of biomembranes subject to ultraviolet irradiation.

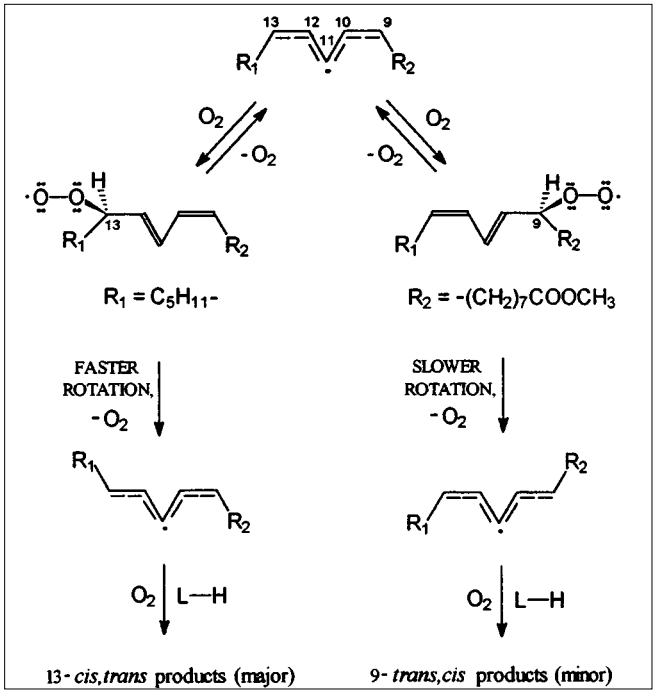

FIG. 8. Schematic pathway leading to hydroperoxide product ratios of 13:9 greater than unity for peroxidation of linoleate in 0.50 M SDS.

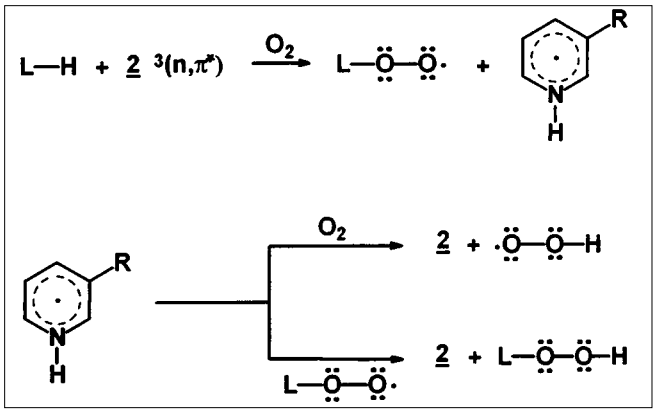

FIG. 9. A pathway for recycling of an azaaromatic during photo-initiated peroxidation.

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Effects of Cationic Liposome–DNA Complexes on Pulmonary Surfactant Function *in vitro* and *in vivo*

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ABSTRACT: Cationic liposome–DNA complexes are being evaluated as potential gene therapy agents for the lung. Cations have strong effects on the biophysical functions of lung surfactant. Therefore, we assessed whether cationic liposomes [composed of *N*-(1-(2,3-dioleyloxy) propyl)-*N,N,N*-trimethyl-ammonium chloride and dioleylphosphatidylethanolamine] with or without DNA affect behavior of four types of surfactant *in vitro*. Experiments were carried out using a modified Wilhelmy surface balance. The ability of surfactants that contain protein and anionic lipids to lower surface tension was inhibited in the presence of cationic liposomes. Inactivation was less when DNA was preincubated with cationic liposomes. Surfactant that contained neither protein nor anionic lipids was not inactivated. Mechanical properties of the lung were studied to assess *in vivo* surfactant function after intratracheal instillation of a cationic liposome–DNA complex into adult rats. Pressure–volume deflation curves were shifted by 18% compared with those from normal (untreated) animals, but this effect was transient and not different from that observed in animals who received a similar volume of saline. These findings indicate that cationic liposomes alone may have deleterious effects on behavior of some surfactants possibly by disrupting charge interactions between negatively charged phospholipids and surfactant proteins. When DNA is added to liposomes before exposure to surfactants, the adverse charge interactions may be obviated by charge neutralization of liposomes by DNA.

Lipids 32, 247–253 (1997).

The lung has unique advantages as a site for somatic cell gene therapy because it can be targeted using intratracheal, intravenous, or aerosol routes (1–6). The entry of DNA into the cell and its subsequent expression has been demonstrated using a variety of transfection techniques that include fusion agents, polycations, neutral, or charged liposomes, viruses with specific tropism for airway epithelial cells, replication-deficient adenovirus, as well as reagents such as calcium phosphate, or DEAE–dextran. Limitations exist with all transfection techniques. Transfection using charged liposomes has been hampered by sonication-induced DNA degradation and low efficiency of cellular uptake and expression. Adenovirus vectors have advantages over liposomes in regard to efficacy of *in vivo* gene transfer; however, this technique may result in low levels of adenoviral gene products that lead to immunogenicity and inflammation in target tissues (7). Cationic liposomes are used to introduce genomic material into cultured cells and into animals. Pulmonary surfactant lines the pulmonary epithelial lumen and is known to be affected by charge interactions. Both positive and negative effects of cations on surface properties of lung surfactant have been shown (8–11). In general, surfactant function requires modest concentrations of mono- or divalent cations and is inhibited by higher concentrations of di- or trivalent cations. Increase in the surface tension at the air/lipid/aqueous interface after addition of these compounds to surfactant has been explained by cation association with anionic constituents of surfactant that may affect the electrostatic formation of cation–phosphatidylcholine bridges and phospholipid–protein interactions (11). In order to determine the salience of charge interactions between cationic liposomes and surfactant, we have studied whether addition of cationic liposomes with or without DNA has adverse effects on surfactant function *in vitro* and *in vivo*.

METHODS

Liposome preparation. Dioleylphosphatidylethanolamine (DOPE) (Avanti Polar Lipids, Alabaster, AL) dissolved in chloroform (10 μg/μL) and *N*-(1-(2,3-dioleyloxy)propyl)-*N,N,N*-trimethylammonium chloride (DOTMA) (Syntex Corp., Palo Alto, CA) dissolved in ethanol were mixed at DOPE/DOTMA (1:1, by weight) and evaporated to dryness on a rotary evaporator. Two–three mL of buffer solution (50 mM Tris, pH 7.4, 0.5 mM EDTA, 50 mM NaCl, 100 μM $ZnCl_2$) were added to bring the lipids to 5 mg/mL, and the mixture was sonicated in a bath sonicator (55 kHz) under argon for 20 min at room temperature (24°C).

Surface tension measurements. Surfactant preparations were diluted with appropriate amounts of buffered normal

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Abbreviations: DOPE, dioleylphophatidylethanolamine; DOTMA, N-(1-(2,3-dioleyloxy) propyl)-*N,N,N*-trimethyl-ammonium chloride; DPPC, dipalmitoylphosphatidylcholine; DS, dog surfactant; pCMVβ, cytomegalovirus promoter β-galactosidease fusion gene plasmid construct.

saline to give a lipid concentration of 0.3 mg/mL and incubated for 16 h at 4°C. Survanta® (Ross Laboratories, Columbus, OH), Curosurf® (Chiesi Laboratories, Parma, Italy), Exosurf® (Burroughs Wellcome Co., Research Triangle Park, NC), or surfactant obtained from alveolar lavage from dogs (DS) by density gradient centrifugation (12) were each mixed with liposomes at surfactant lipid/liposome lipid (50:1, 10:1, 5:1, 1:1, by weight) then incubated at room temperature for 30 min. Experiments were repeated with plasmid DNA, cytomegalovirus promoter β-galactosidase fusion gene plasmid construct (pCMVβ), added to the mixtures at a liposome/DNA ratio of 5:1 by weight. This ratio was based on optimal ratios used in transfection experiments by us and others (13,14).

A Wilhelmy-Langmuir surface balance at room temperature was used to determine the surface tension-lowering properties of each of the above preparations *in vitro*. Test samples were applied to the surface or into the subphase of 30 mL saline in the Wilhelmy balance, allowed to absorb for 2 min, and minimum surface tension changes were recorded at the first and fifth compression according to previously published methods (15). To ensure that the observed effects were not due to nonspecific lipid chemistry, we repeated the experiments with Survanta® and DS at 37°C. (Others have indicated that performing analyses at either 22 or 37°C does not qualitatively affect the properties of lipid–protein monolayers (16) since the gel-to-liquid crystalline transition for dipalmitoylphosphatidylcholine (DPPC) is 41°C.

Pressure–volume relationships. Adults rats (340–450 g) were anesthetized with 90 mg/kg ketamine (Fort Dodge Laboratories, Fort Dodge, IA) and 10 mg/kg xylazine (Rugby Laboratories, Rockville Centre, NY) given intraperitoneally. Direct tracheal intubation was carried out using an 18-gauge Angiocath® (Becton-Dickinson, Franklin Lakes, NJ) after visualizing the glottis by means of a laryngoscope (Miller 0). Animals were randomly assigned to three groups. The first group (n = 10) was instilled with liposome–DNA preparation, the second group (n = 10) was instilled with saline, and the third group (n = 10) was lavaged with saline.

A solution containing 500 μg liposome (250 μg DOTMA, 250 μg DOPE) complexed with 100 μg DNA (pCMV β; galactosidase cDNA) made up to a total volume of 1 mL with saline (pH 7.0) was incubated at room temperature for 15 min then instilled in the trachea of animals in the first group as a bolus. Dosage of liposome–DNA was based on the following considerations. Adult rat lung contains an estimated 10–15 mg/kg body weight surfactant lipid (17). Rats weighed 340–450 g. Therefore, liposomes were given at a dose of 500 μg to achieve an estimated endogenous surfactant lipid/liposome ratio of 10:1 by weight. Plasmid DNA was added to the liposome preparation at a 1:5 mass ratio (0.00003:1 mole/mole), as explained previously in this article. The DNA/lipid charge ratio (–/+) was determined to be 1:1.11.

The second group was given a similar volume of normal saline delivered into the trachea. This volume was determined in separate experiments to optimize gross dispersion throughout the lung using either Coomasie brilliant blue or [^{14}C]-L-3-phosphatidylcholine, 1,2-di[^{14}C]oleoyl) as indicators of distribution. The dye and the radioactive signal were seen equally in both lungs, out to the periphery. Studies to identify cell type were not done.

Animals in the third group underwent pulmonary lavage using an aliquot of saline (10 mL) instilled and withdrawn from the trachea four times. After instillation of cationic liposome–DNA mixtures or saline, all animals breathed room air and were allowed to fully recover from anesthesia for either 3 1/2 h or for 6 h, at which time they were killed with a lethal (200 mg/kg intraperitoneal) dose of pentobarbital. Two time points were chosen to assess the reversibility of the potential effects of cationic liposome–DNA mixtures upon lung function, as determined by pressure–volume deflation curve analysis. Lungs and heart were removed en bloc, a 5 French vinyl catheter was secured in the trachea, and the lungs were degassed at a pressure of not less than 20 torr in an evacuated bell jar. Next, a pressure transducer (Gould® Windograf; Gould Instrument Systems, Valley View, OH) was used to monitor pressure changes in the lungs while lungs were manually inflated with an airtight syringe to a maximum of 30 cm H_2O at room temperature. Maximum inflating pressure was maintained for 1 min. The lungs were then deflated in steps over approximately 2 min while recording quasistatic pressure and volume changes. Lung volumes were corrected for gas compression in the syringe and tubing. Total lung capacity was defined as lung volume after 1 min at 30 cm H_2O pressure.

Data analysis. Results are expressed as mean ± standard error of the mean. Intergroup comparisons were done by unpaired t-test. Statistical significance was accepted as $P < 0.05$.

RESULTS

Surface tension measurements on modified Wilhelmy balance. Neither incubation with up to 37.5 μg DOTMA/DOPE nor addition of DNA to the liposomes changed the surface properties of 37.5 μg Exosurf (Fig. 1). We found an impairment of the surface tension-lowering properties of the three lung-derived surfactant preparations when mixed with cationic liposomes. Significant inactivation (defined as minimum surface tension >10 mN/m) was evident starting at a surfactant lipid/cationic lipid ratio of 10:1 for Survanta®, Curosurf®, and DS (Figs. 2–6). A squeeze-out phenomenon was not seen, and there were no differences in the above findings when the mixtures were applied to the subphase instead of the surface of the trough. Minimum surface tensions at 25°C obtained with Curosurf®, Survanta®, and DS were 3 ± 0.5 mN/m (n = 5), 7 ± 0.1 mN/m (n = 5), and 2 mN/m (n = 1), respectively (Figs. 2–4). When the surfactants were combined with liposomes at 1:1 (g lipid/g lipid) ratio, these values increased to 16 ± 0.6 mN/m (n = 5), 15 ± 3 mN/m (n = 5) ($P <$ 0.05), and 15 mN/m (n = 1). However when surfactant, cationic lipids, and DNA were mixed together, at a 1:1:0.2 (g lipid/g lipid/g DNA) ratio, minimum surface tensions of Curosurf®, Survanta®, and DS were 9 ± 1 mN/m (n = 5), 12 ± 0.4 mN/m (n = 5) ($P < 0.05$), and 14 mN/m (n = 1), respectively. The

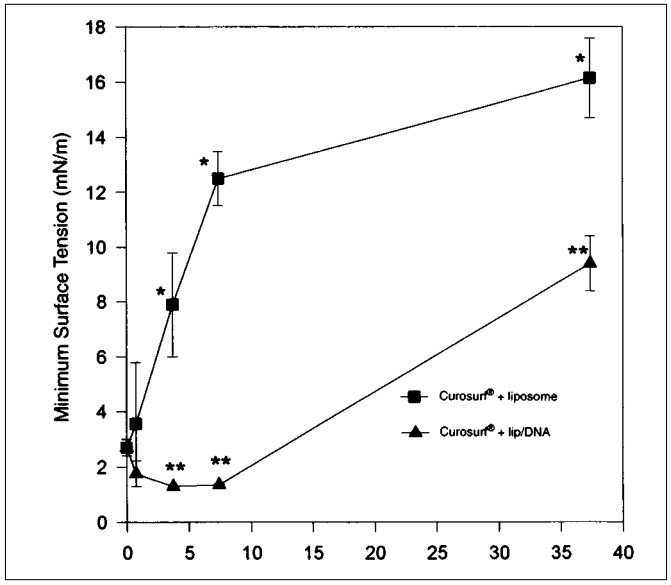

FIG. 1. Minimum surface tensions at 25°C of Exosurf® (Burroughs Wellcome, Research Triangle Park,) on Wilhelmy balance when mixed with cationic liposomes. A constant amount of 37.5 μg surfactant lipid was mixed with varying amounts of liposome (lip) *N*-(1-(2,3-dioleyloxy)propyl)-*N,N,N*-trimethylammonium chloride/dioleylphosphatidylethanolamine (DOTMA/DOPE) to give ratios of (by mass lipid) 50:1, 10:1, 5:1, and 1:1 (■). 37.5 μg surfactant lipid was mixed with increasing amounts of 5:1 (w/w) liposome/DNA complex (▲). Results at the fifth compression are shown. Data are means of between three and eight different experiments. Inactivation by DOTMA/DOPE was not significant.

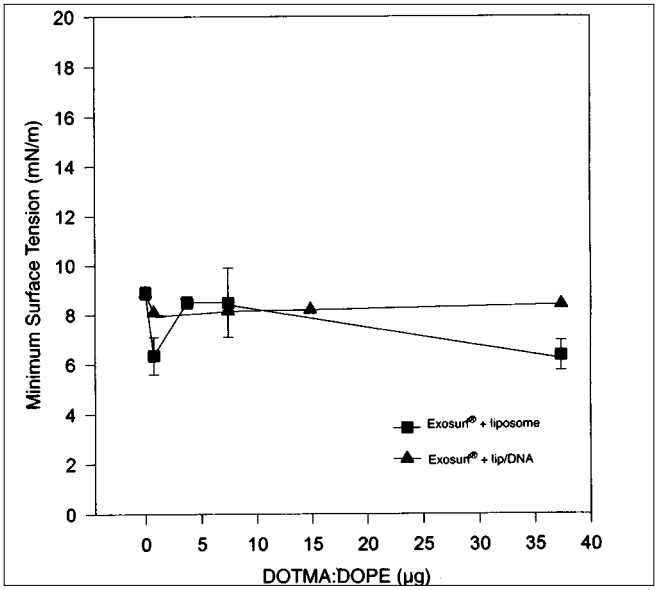

FIG. 2. Minimum surface tensions at 25°C of Curosurf® (Chiesi Laboratories, Parma, Italy) on Wilhelmy balance when mixed with cationic liposomes. A constant amount of 37.5 μg surfactant lipid was mixed with varying amounts of liposome (DOTMA/DOPE) to give ratios of (by mass lipid) 50:1, 10:1, 5:1, and 1:1 (■). 37.5 μg surfactant lipid was mixed with increasing amounts of 5:1 (w/w) liposome/DNA complex (▲). Results at the fifth compression are shown. Data are means of between three and eight different experiments. Inactivation by DOTMA/DOPE was significant starting at a ratio of 10:1 (*: $P < 0.05$). Addition of DNA decreased surfactant inactivation significantly (**: $P < 0.05$ compared with DOTMA/DOPE). See Figure 1 for abbreviations.

amount of liposome required to cause an increase in the surface tension to > 10 mN/m was approximately six times more in the presence of DNA.

Similar effects on minimum surface tension were obtained when Survanta® or DS were analyzed with cationic liposomes or cationic liposome–DNA complexes at 37°C (Figs. 5,6), substantiating the above findings.

Pressure–volume curves. Deflation stability of the rat lung was evaluated 3 1/2 or 6 h following tracheal instillation by comparing the gas volumes retained in the resected lungs at 5 cm H_2O pressure on the deflation limb of the curve. Deflation stability at 3 1/2 h in liposome–DNA-treated lungs at 5 cm H_2O was 18% lower than the mean for the untreated lungs ($P < 0.05$); however, a similar change from untreated controls was seen also in lungs from saline-treated rats (Fig. 7). This effect seen in the liposome–DNA group was transient since pressure–volume curves measured at a later time (6 h) were close to normal (Fig. 8).

DISCUSSION

Surface tension activity of the synthetic pulmonary surfactant Exosurf® was unaffected either by cationic liposomes or by cationic liposome–DNA mixtures. Our major findings support the notion that cationic liposomes affect the function of Curosurf®, Survanta®, and DS. Addition of DNA to cationic liposomes reduces the ability of liposomes to inactivate Curosurf®, Survanta®, and DS. That is, the addition of DNA to cationic liposomes increases the amount of liposome needed to cause inactivation of these surfactants, possibly by neutralizing liposome charge. Cationic liposome–DNA mixtures have transient effects on lung function *in vivo* that are no greater than those seen with saline alone.

We found that lung-derived surfactants were nevertheless susceptible to some inactivation *in vitro* by cationic liposome–DNA mixtures when the cationic lipid/surfactant lipid ratio was greater than 1:5 in the case of Curosurf® and Survanta®, or when cationic liposome lipid/surfactant lipid ratios were greater than 1:10 in the case of DS. We used an estimated cationic lipid/lung surfactant lipid ratio of 1:10 *in vivo*, primarily since this was the approximate amount that other workers had used in tracheal instillation of liposome–DNA mixtures to achieve *in vivo* transfection (1–3,5,6,13,23,24,26). We found that these amounts of liposome–DNA had no significant effect upon lung function, as measured by lung pressure–volume changes, when compared with instillation of a bolus of saline. Future investigators who might wish to enhance transfection efficiencies by treatment with much greater amounts of cationic liposomes may cause inactivation of the endogenous surfactant, followed by loss of lung function. These inactivation effects might be reduced if cationic liposome–DNA mixtures were to be instilled in the presence of an inert surfactant,

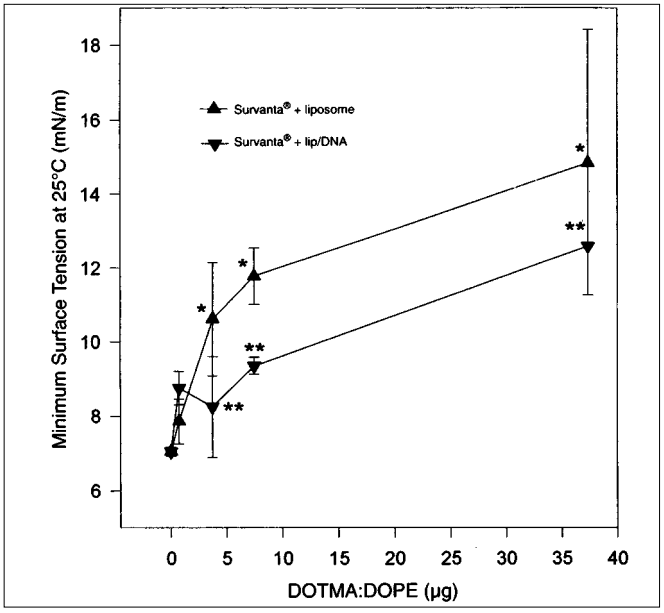

FIG. 3. Minimum surface tensions at 25°C of Survanta® on Wilhelmy balance when mixed with cationic liposomes. A constant amount of 37.5 µg surfactant lipid was mixed with varying amounts of liposome (DOTMA/DOPE) to give ratios of (by mass lipid) 50:1, 10:1, 5:1, and 1:1 (△). 37.5 µg surfactant lipid was mixed with increasing amounts of 5:1 (w/w) liposome/DNA complex (hatched triangle). Results at the fifth compression are shown. Data were mean of between three and eight different experiments. Inactivation by DOTMA/DOPE was significant starting at a ratio of 10:1 (*: $P < 0.05$). Addition of DNA decreased surfactant inactivation significantly (**: $P < 0.05$ compared with DOTMA/DOPE). See Figure 1 for abbreviations.

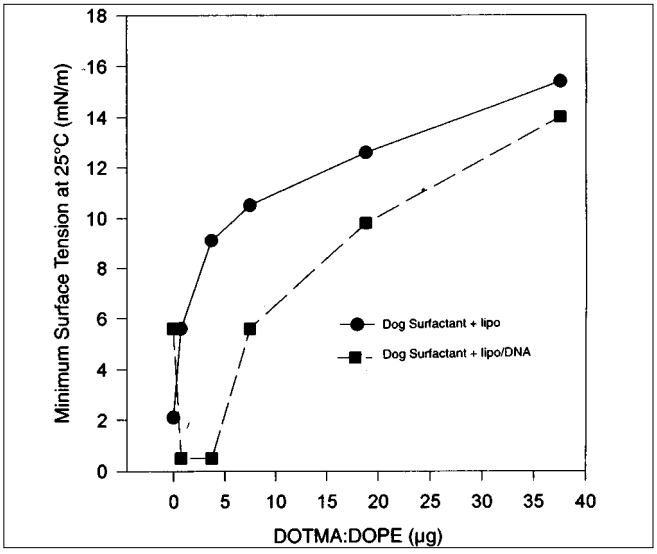

FIG. 4. Minimum surface tensions at 25°C of dog surfactant on Wilhelmy balance when mixed with cationic liposomes. A constant amount of 37.5 µg surfactant lipid was mixed with varying amounts of liposome (DOTMA/DOPE) to give ratios of (by mass lipid) 50:1, 10:1, 5:1, 2:1, and 1:1 (●). 37.5 µg surfactant lipid was mixed with increasing amounts of 5:1 (w/w) liposome/DNA complex (■). Results at the fifth compression are shown. Data were from a single experiment of one extract. Replicate experiments gave similar results. Dog surfactant was inactivated by DOTMA/DOPE; this inactivation was partly reversible by addition of DNA. See Figure 1 for abbreviations.

such as Exosurf®. Results from our laboratory suggest that these assumptions are correct (13).

Various alternative explanations are possible. We may have underestimated the surfactant-to-liposome ratio that exists in our *in vivo* experiments for our *in vitro* experiments. Others have found that increasing surfactant-to-inactivator ratio reduces the degree of surfactant dysfunction. Alternatively, liposome inactivation of surfactant may occur in mixing experiments, but not when surfactant preexists as a surface film at high surface pressure as would be the case in normal lungs. In addition, if this reasoning is correct, then liposome–DNA mixtures might have a greater than expected adverse effect in lungs with preexisting damage, that is to say, the sort of lungs in which gene therapy might be considered.

Susceptibility of various surfactants to inactivation *in vitro* by cationic liposomes varies. Survanta is a natural bovine lung extract containing phospholipids, neutral lipids, fatty acids, and surfactant-associated proteins to which dipalmitoylphosphatidylcholine (DPPC), palmitic acid, and tripalmitin are added. Curosurf is made from minced pig lung by chloroform–methanol extraction and liquid–gel chromatography. It contains saturated phosphatidylcholine along with other phospholipids and proteins, but no triglycerides, cholesterol, or free fatty acids. Exosurf® is a synthetic, protein-free surfactant composed of DPPC, hexadecanol (cetyl alcohol), and tyloxapol, a detergent that disperses DPPC. Hexadecanol is metabolized to palmitic acid which is later utilized in the synthesis of phospholipids. It may serve the same function in Exosurf® as palmitic acid does in Survanta®. Palmitic acid in natural lung surfactant is claimed to fluidize and separate the normally rigid DPPC bilayers in solution to enhance dispersion of the surfactant and increase absorption to interfaces (18). Previous work indicates that Survanta® and Curosurf® are more resistant to inactivation by albumin, fibrinogen, and hemoglobin when compared with Exosurf® when assessed by the pulsating bubble surfactometer (19). Our experiments, using a different measurement of inactivation (Wilhelmy balance), indicate that DS, Curosurf®, and Survanta® are all inactivated to the same degree in the presence of cationic liposomes, but Exosurf® is not. Exosurf® differs from the animal-derived surfactants in two major respects; it lacks surfactant-specific proteins and it lacks anionic lipids. Because the addition of negatively charged DNA to DS, Curosurf® and Survanta® reduces the liposome inactivation observed in the Wilhelmy balance experiments, we conclude that liposome–surfactant charge interaction is most likely responsible for the inactivation observed. These results are consonant with previous work that indicates charge interactions are potent inactivators of surfactant function *in vitro* (11,20). The ability of the cationic liposomes to fuse with negatively charged liposomes is strongly reduced in the presence of DNA (21). It is also possible that the *in vitro* measures on the Wilhelmy balance are more sensitive in detecting changes in surface activity than the measure of pressure–volume relation-

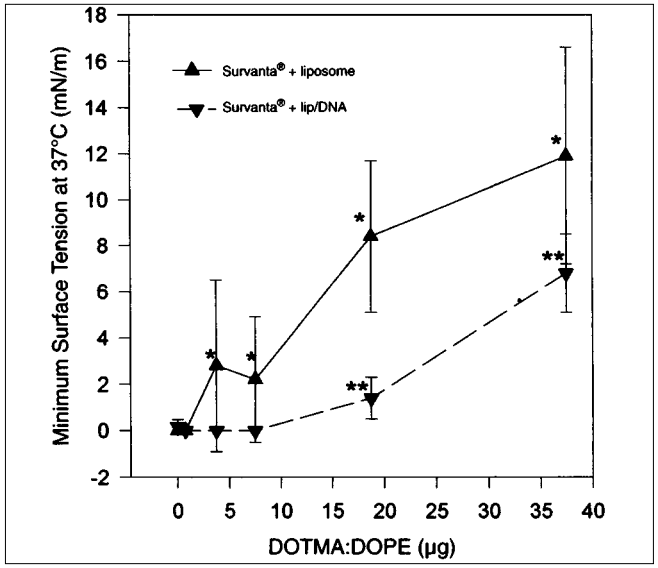

FIG. 5. Minimum surface tensions at 37°C of Survanta® on Wilhelmy balance when mixed with cationic liposomes. A constant amount of 37.5 µg surfactant lipid was mixed with varying amounts of liposome (DOTMA/DOPE) to give ratios of (by mass lipid) 50:1, 10:1, 5:1, 2:1, and 1:1 (Δ). 37.5 µg surfactant lipid was mixed with increasing amounts of 5:1 (w/w) liposome/DNA complex (▽). Results at the fifth compression are shown. Data were mean of three different batches. Inactivation by DOTMA/DOPE was significant starting at a ratio of 50:1 (* : $P < 0.05$). Addition of DNA decreased surfactant inactivation significantly (** : $P < 0.05$). See Figure 3 for company source. See Figure 1 for abbreviations.

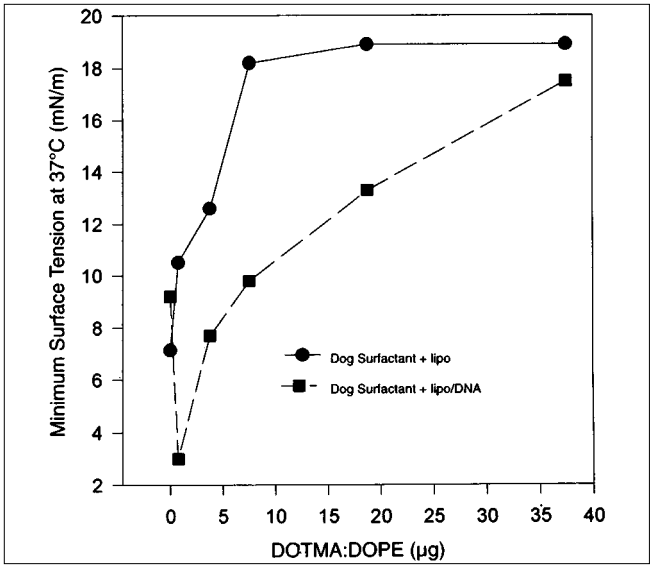

FIG. 6. Minimum surface tensions at 37(C of dog surfactant on Wilhelmy balance when mixed with cationic liposomes. A constant amount of 37.5 (g surfactant lipid was mixed with varying amounts of liposome (DOTMA:DOPE) to give ratios of (by mass lipid) 50:1, 10:1, 5:1, 2:1, and 1:1 (filled circles). 37.5 (g surfactant lipid was mixed with increasing amounts of 5:1 (w/w) liposome:DNA complex (filled squares). Results at the fifth compression are shown. Data were from a single experiment of one extract. Replicate experiments gave similar results. Dog surfactant was inactivated by DOTMA:DOPE; this inactivation was partly reversible by addition of DNA.

ships *in vivo*. Finally, although we carried out distribution experiments, it is possible that microdistribution of liposome–DNA was not widespread enough for *in vivo* changes to be detected.

The modest and transient effects on pulmonary mechanics of liposome–DNA mixtures are no greater than effects found with saline alone. These transient effects on mechanical properties of the lung most likely are secondary to the volume load of 1 mL (2.5–3 mL/kg) that could decrease total lung capacity by about 20%. Alternatively, inflammation with saline has been observed as early as 4 h after instillation into swine and rat lungs (6,22).

Others have studied whether liposomes used for DNA delivery affect lung function. Recently Canonico and coworkers (23) have shown that repeated weekly intravenous or aerosol administration of plasmid DNA complexed to cationic liposomes resulted in no adverse effects on pulmonary histology, gas exchange, compliance, or resistance over a 4-wk period when given to rabbits in an aerosol or intravenous solution (2500 µg liposome and 500 µg DNA in a total volume of 2.5 mL per 1.0–1.3 kg rabbit). Our study differs from theirs because we assessed the short-term effects of liposomes when administered as a bolus intratracheally. We used a dose of liposome–DNA similar to that used by Canonico and coworkers (23) and administered in a slightly larger volume: 1 mL per 0.3–0.4 kg rat. In other experiments meant to assess potential toxic side effects, liposomes have been tested on human volunteers in an aerosolized form (24). There were no changes in oxygen saturation or the spirometric values at 1 h after administration. Cationic liposome as a delivery agent for the human cystic fibrosis transmembrane conductance gene has no effects on spirometric values or the nasal potential difference in human subjects treated intranasally (25). In general, our results agree with these previous studies in that cationic liposome–DNA mixtures delivered by the airway appear to have transient and probably inconsequential effects.

A recent approach in pulmonary gene therapy has been to admix the liposome–DNA complex with pulmonary surfactant (13,26). The transfection efficiency with adenovirus increased when mixed with Survanta® before being instilled into the airways (26). Exosurf® mixed with DOTMA/DOPE–DNA preparations results in a fivefold increase in transfection efficiency, using cationic liposome–DNA preparations alone (13).

In summary, our *in vitro* experiments indicate that cationic liposomes with or without DNA affect different surfactants differently. Results imply that charge interactions are important for adequate surfactant function *in vitro*. Cationic liposome–DNA mixtures that contain up to 500 µg of cationic liposome per 0.3–0.4 kg weight (1.6 g/kg) with a liposome/DNA ratio of 5:1 exhibit effects on pulmonary mechanics that are transient and are no greater than those seen with saline alone. These results and those of others indicate that cationic liposome–DNA delivery to the lungs will not ad-

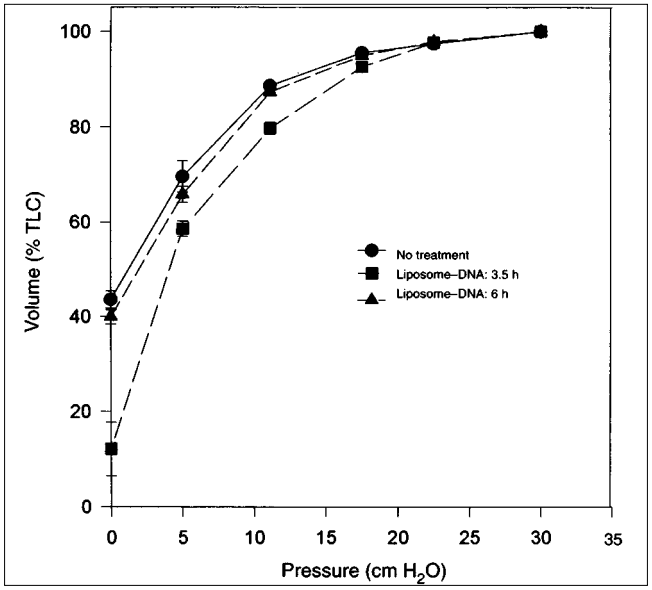

FIG. 7. Pressure–volume deflation curves in control (filled hexagons), liposome/DNA-treated (●), saline-treated (■), and lavaged rats (▲). Lungs were isolated 3.5 h following treatment. The difference between the liposome/DNA- or saline-treated lungs was not significant ($P > 0.1$). Volume is expressed as percentage of total lung capacity (TLC).

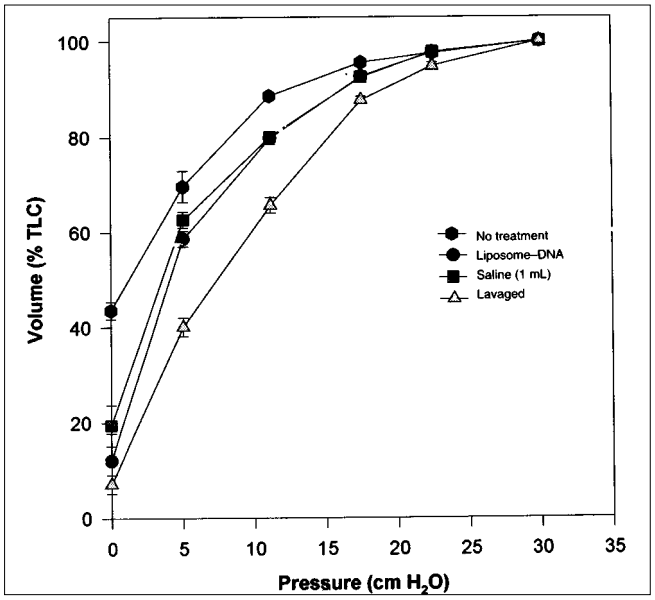

FIG. 8. Pressure–volume deflation curves in control (●), liposome/DNA at 3.5 h (■), and 6 h (▲) following tracheal instillation. The difference between control or liposome/DNA-treatment 6 h following instillation was not significant ($P > 0.1$). Volume is expressed as percentage of total lung capacity (TLC).

versely affect surfactant function in normal lungs where the amounts of monovalent cationic lipid do not exceed 10% (w/w) of estimated pulmonary surfactant lipid.

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Simvastatin Impairs Mitogen-Induced Proliferation of Malignant B-Lymphocytes from Humans—*in vitro* and *in vivo* Studies

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ABSTRACT: Chronic lymphocytic leukemia (CLL) cells express lower low density lipoprotein (LDL) receptor activity and higher 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) reductase activity than normal mononuclear blood cells indicating that CLL cells may depend on cholesterol synthesis for their proliferation. We studied the effects of competitive inhibitors of HMG–CoA reductase on malignant lymphocyte proliferation *in vitro* and *in vivo*. Tumor B-cells from 13 patients with CLL, hairy cell leukemia, or immunoblastic B-cell lymphoma were cultured for 4 d in the presence of B-cell mitogens and cholesterol synthesis inhibitors. Simvastatin and lovastatin suppressed, in a concentration-dependent manner, the mitogen-induced cellular thymidin uptake in medium with 10% human AB-serum or lipoprotein-deficient serum. Pravastatin was active only in medium with lipoprotein-deficient serum. Ten previously untreated patients with CLL received simvastatin orally, 40 mg daily for 12 wk. Mean reductions in total plasma and LDL cholesterol were 30% (range 9–46%) and 37% (range 16–63%), respectively. Cells from four patients showed moderate to minor increases in the degradation rate of ^{125}I-LDL suggesting that the need for exogenous cholesterol had increased, three patients showed an increase in HMG–CoA reductase activity, and the cells from one patient showed both. There was no significant change in the clinical disease status during medication. However, four of the ten patients developed a therapy-demanding progressive disease during the subsequent year. Further clinical studies with cholesterol synthesis inhibitors in leukemia are warranted.

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Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world. It affects elderly people, median age at diagnosis is about 65 yr, and about 10% of the patients are diagnosed before the age of 50 yr. The CLL cell is a B-lymphocyte with a mature appearance, and it coexpresses B-cell markers and the pan-T-cell marker CD5. Most patients with CLL do not require therapy at diagnosis, but subsequently progressive disease is common. The median therapy-free survival is about 4 yr. First-line therapy with alkylating agents usually ameliorates symptoms, but often drug resistance develops. Infections and cytopenias are the major clinical problems in advanced disease. The median survival of about 80 mon is significantly shorter than age- and sex-matched controls (1).

Proliferating cells have a demand for cholesterol for membrane synthesis. The cellular cholesterol requirements are met by receptor-mediated uptake of low density lipoprotein (LDL) and/or by endogenous synthesis of cholesterol, the rate-limiting enzyme in the synthesis pathway being 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) reductase (2). LDL is the major cholesterol-carrying lipoprotein in human plasma, containing approximately 70% of total plasma cholesterol. Once bound to the specific LDL receptor, LDL is internalized and degraded in lysosomes. The released cholesterol is available for incorporation into cellular membranes or stored in reesterified form (2). Cellular cholesterol requirements may be increased in acute myelogenous leukemia, since leukemic cells from such patients have elevated receptor-mediated uptake of LDL as compared with white blood cells from healthy individuals (3,4).

CLL cells have a reduced ability to form LDL receptors (5), whereas HMG–CoA reductase activity is elevated (6), indicating that CLL cells may depend on cholesterol synthesis for their proliferation. If so, pharmacological suppression of cholesterol synthesis should prevent proliferation of malignant B-lymphocytes. This study was undertaken to test this hypothesis, utilizing both *in vitro* and *in vivo* experiments.

METHODS

Growth inhibitory effects of HMG–CoA reductase inhibitors in vitro: cells. For the *in vitro* studies, cells from 13 patients with B-cell malignancies were used, 11 with CLL, 1 with immunoblastic B-cell lymphoma, and 1 with hairy cell leukemia. Mononuclear cells were isolated from peripheral blood by centrifugation on Lymphoprep (density 1.077 g/mL; Nyegaard A/S, Oslo, Norway) and washed with ice-cold phosphate buffered saline (140 mmol/L NaCl, 2.7 mmol/L KCl, 9.5 mmol/L Na_2HPO_4, and 9.5 mmol/L KH_2PO_4, pH

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Abbreviations: CLL, chronic lymphocytic leukemia; HMG–CoA, 3-hydroxy-3-methylglutaryl–CoA; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum.

7.4) as described (5). All cell samples were analyzed for B-cell clonality by surface immunoglobulin staining.

Culture conditions. The cells were cultured at a concentration of 2×10^6/mL in Eagle's medium containing 10% heat-inactivated human AB-serum or 10% human lipoprotein-deficient serum (LPDS) prepared as previously described (4). Triplicate cultures of 0.2 mL were set up in 96-well microplates (Falcon; Becton, Dickinson and Co., Oxnard, CA) and incubated at 37°C in humified air containing 5% CO_2. CLL cells can be induced to proliferate *in vitro* by polyclonal B-cell activators, i.e., ligands with a capability of activating normal B-cells without utilizing the specific B-lymphocyte receptor, the immunoglobulins. We here used tetradedecanoyl-phorbol-13-acetate (2 μmol/L; Sigma Chemical Co., St. Louis, MO) or pokeweed mitogen (5 μg/mL; Sigma Chemical Co.), which was added to the cultures with or without the competitive HMG–CoA reductase inhibitors lovastatin (former name mevinolin), simvastatin, or pravastatin, each at the concentrations 2 μg/mL and 20 μg/mL.

1.0 μ Ci of tritium-labelled thymidine (Radiochemical Centre, Amersham, United Kingdom) was added to the microplate cultures on day 3. Harvesting was performed on day 4 using a Skatron Harvesting Machine (Lierbyen, Norway), and the radioactivity was measured with a scintillation spectrophotometer (Intertechnique, Nanoteknik, Sweden).

Statistical analyses. A paired ratio *t*-test, with the *t*-statistic mean−1/SD/$\sqrt{n}$ was used to test for differences in mitogen-induced thymidine uptake and drug effects in cell cultures with two types of sera. In the first case, a ratio between the net cpm in each type of sera was calculated for each paired sample (Table 1). In the latter, the ratios between mitogen-induced thymidine uptake with and without cholesterol synthesis inhibitors were first calculated in each type of sera wherafter the ratios from paired samples were compared with the paired ratio *t*-test (Table 2). A mean ratio significantly different from 1 denotes a significant difference in thymidine uptake between the two types of culture conditions or different drug efficacy in the two types of sera. A *P*-value ≤0.05 was considered as statistically significant.

In vivo *study: patients.* Ten patients with previously untreated B-cell CLL were given simvastatin (Zocord®; Merck, Sharp and Dohme, Stockholm, Sweden), 40 mg daily by a single oral dose for twelve weeks. Clinical characteristics of the patients are given in Table 3. The patients underwent blood and serum chemistry analysis and clinical examination twice monthly during medication, and at least every third month prior to and after the therapy period. The study was approved by an Institutional Review Board.

Lipoproteins. LDL (density 1.020–1.063 g/mL) and human LPDS (density >1.215 g/mL) were isolated from serum of healthy blood donors by sequential ultracentrifugation (7). The purity of LDL and LPDS preparations was examined by agarose gel electrophoresis, and the absence of cholesterol in LPDS was confirmed by enzymatic cholesterol analysis (Merck, Darmstadt, Germany). ^{125}I-labeled LDL (specific activity 140–260 cpm/ng protein) was prepared as described by Langer *et al.* (8). Less than 1% of the radioactivity in the ^{125}I-LDL preparations was present as free iodide. The concentrations of LDL refer to protein.

LDL receptor and HMG–CoA reductase determinations. Mononuclear cells from peripheral blood were isolated as described above. An aliquot of the washed cells was used for immediate determination of LDL receptor activity. The high affinity (receptor-mediated) degradation rate of ^{125}I-LDL was used as a measure of LDL receptor activity (4). In brief, 3×10^6 leukemic cells were incubated with 25 μg of ^{125}I-LDL at 37°C in 1 mL of RPMI 1640 supplemented with 10% LPDS. Cellular degradation of ^{125}I-LDL was determined from the formation of acid-soluble radioactivity in the incubation medium. The high affinity degradation rate was calculated by subtracting the degradation of ^{125}I-LDL in the presence of a 20-fold excess of unlabeled LDL (unspecific degradation) from the degradation in the absence of unlabeled LDL (total degradation). Cells were also subjected to a 48-h incubation in medium containing 10% LPDS so as to study the induction of LDL receptor activity. Cells were plated at a concentration of 3×10^6 cells/mL in 1 mL, and after 48-h cellular LDL receptor activity was determined as described above without

TABLE 1
Mitogen-Induced Thymidine Uptake (cpm) with and without Inhibitors of HMG–CoA Reductase[a]

	Standard human AB serum	Lipoprotein-deficient serum	LPDS/standard serum ratio	
Culture	Median cpm (range)	Median cpm (range)	Mean ± SD	*P*-value[b]
Control	640 (300–2,050)	640 (480–870)	0.82 ± 0.36	n..s.
M	16,260 (1,450–69,580)	8,920 (1,680–34,310)	0.63 ± 0.36	<0.01
M + Pra2	14,285 (2,140–75,990)	8,340 (1,340–37,750)	0.58 ± 0.38	<0.01
M + Pra20	14,600 (1,000–80,430)	3,380 (1,010–23,520)	0.47 ± 0.31	<0.001
M + Lov2	9,450 (800–55,980)	5,210 (410–22,440)	0.44 ± 0.35	<0.001
M + Lov20	1,180 (140–5,680)	680 (280–3,930)	0.63 ± 0.46	<0.05
M + Sim2	4,345 (1,240–41,170)	3,880 (510–16,160)	0.63 ± 0.45	<0.05
M + Sim20	975 (350–5,240)	600 (90–1,610)	0.58 ± 0.27	<0.001

[a]In 4-day cell cultures with standard serum (n = 13) and with lipoprotein-deficient serum (n = 11), and ratio between the two culture conditions. M, Mitogen; Pra, pravastatin; Lov, lovastatin; Sim, simvastatin; 2 and 20 denote drug concentration in μg/mL.

[b]Paired ratio *t*-test with the *t*-statistic mean−1/SD/$\sqrt{n}$; n.s., not significant; HMG–CoA, 3-hydroxy-3-methylglutaryl–CoA; LPDS, lipoprotein-deficient serum.

TABLE 2
Ratio Between Mitogen-Induced Thymidine Uptake with and without Inhibitors of HMG–CoA Reductase[a]

	Standard human AB serum	Lipoprotein-deficient serum	LPDS/standard serum ratio	
Culture	Median (range)	Median (range)	Mean ± SD	*P*-value
Pra2	1.03 (0.85–2.17)	0.79 (0.38–1.81)	0.82 ± 0.30	n..s.
Pra20	0.99 (0.64–2.05)	0.68 (0.17–1.01)	0.68 ± 0.27	<0.005
Lov2	0.65 (0.039–1.02)	0.38 (0.12–1.68)	0.71 ± 0.59	n.s.
Lov20	0.07 (0.046–0.36)	0.07 (0.014–0.17)	1.22 ± 0.95	n.s.
Sim2	0.47 (0.14–1.44)	0.27 (0.11–0.74)	1.13 ± 0.89	n.s.
Sim20	0.06 (0.034–0.29)	0.05 (0.013–0.25)	1.02 ± 0.48	n.s.

[a]In 4-day cell cultures with standard serum (n = 13) and with lipoprotein-deficient serum (n = 11), and ratio between these ratios. Abbreviations and statistics as in Table 1.

TABLE 3
Clinical Data for 10 Patients with CLL at Baseline (week 0) and Following 12 Wk of Treatment with Simvastatin 40 mg/d. WBC Counts Are Also Given at 3 Mon Poststudy (week 24)[a]

No.	Age/sex	Rai stage	Binet stage	WBC × 10^9/L (% lymphocytes) week 0, week 12, and week 24, respectively	CLL-associated symptoms at baseline	Clinical findings at baseline (CT scan abdomen, physical examination)	Clinical findings at week 12	Reduction in total plasma and LDL-cholesterol at week 12 (% of baseline value)
1	61/M	II	B	200(96) 278(98) 349(99)	Fatigue, sweats	Hepatosplenomegaly, lymphadenopathy	Head and neck lymphadenopathy slight regression	8.7 35.3
2	62/M	II	B	297(98) 346(96) 261(99)	None	Splenomegaly, slight lymphadenopathy	Head and neck lymphadenopathy slight progression	15.9 15.6
3	66/F	I	A	15.9(76) 29.9(82) 29.3(73)	None	Lympadenopathy (CT scan abdomen)	Unchanged	25.5 30.8
4	72/F	II	B	17.6(80) 20.0(85) 18.6 (86)	None	Splenomegaly (not palpable), lymphadenopathy	Head and neck lymphadenopathy slight regression	35.6 36.4
5	74/M	0	A	28.6(82) 31.0(88) n.d.	None		Unchanged	36.5 40
6	44/F	I	B	105(94) 176(95) 50.7(88)	Fatigue	Lymphadenopathy	Unchanged	24.0 30.8
7	67/F	II	A	55.3(93) 62.4(93) 53.5(93)	None	Splenomegaly	Unchanged	33.3 30.6
8	69/M	I	A	38.1(91) 52.7(90) 57.5(90)	None	Hepatomegaly, lymphadenopathy	No hepatomegaly	37.7 40.6
9	67/M	0	A	60.3(96) 55.4(96) 72.6(98)	None		Unchanged	38.6 45.8
10	64/M	I	A	52.4(91) 62.0(88) 73.8(90)	None	Slight lymphadenopathy	Slight progression	45.9 63.0

[a]CLL, chromic lymphocytic leukemia; LDL, low density lipoprotein; WBC, white blood cell.

changing the medium (5). HMG–CoA reductase activity in cell-free extracts was determined from the rate of conversion of 3-hydroxy-3-methyl(3-^{14}C)glutaryl CoA to ^{14}C-mevalonate in detergent-solubilized extracts (9). HMG–CoA reductase activity is expressed as picomoles of ^{14}C-mevalonate formed per min per mg of detergent-solubilized protein. Protein concentration was determined with bovine serum albumin as standard (10).

Plasma lipid analysis. Lipoprotein quantitation was performed by a combination of ultracentrifugation and precipitation (11,12). Briefly, plasma obtained under fasting conditions was spun at 35.000 rpm for 18 h at 4°C in an ultracentrifuge (Centrikon T-2060; Contron Roche, Zurich, Switzerland) equipped with a 45.6 rotor. The tubes were sliced, and the supernatant as well as the infranatant were analyzed for cholesterol and triglyceride content using standard enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany). A portion of the infranatant was treated with phosphotungstic acid in order to precipitate apo B-containing lipoproteins.

RESULTS

Effects of HMG–CoA reductase inhibitors in vitro. The mitogens always induced cell proliferation, as indicated by an increased thymidine uptake in cell cultures. The proliferative results were similar in malignant B-cells from patients with CLL as compared to those from patients with other B-cell malignancies, and the data were therefore pooled. The mitogen-induced thymidine uptake was significantly lower in cells cultured in LPDS compared to those in standard human serum (Table 1), whereas the difference as regards the unstimulated thymidine uptake between cultures in media with the different sera was not significant.

Lovastatin and simvastatin reduced the mitogen-induced thymidine uptake in a concentration-related manner; the thymidine uptake was significantly more reduced at 20 μg/mL than at 2 μg/mL. The thymidine uptake in cultures with LPDS was lower than in cultures with standard serum (Table 1). However, the inhibitory effect of lovastatin and simvastatin on the mitogen-induced thymidine uptake was similar in cultures with serum with and without lipoproteins, as indicated by the ratios in Table 2.

Simvastatin was a more potent inhibitor of mitogen-induced thymidine uptake than lovastatin (20 μg/mL: $P < 0.01$; 2 μg/mL: not significant, Student's *t*-test for paired observations), whereas both simvastatin and lovastatin were more effective than pravastatin (20 μg/mL: both $P < 0.01$; 2 μg/mL: both $P < 0.05$).

Pravastatin did not significantly reduce the mitogen-induced thymidine uptake in standard serum cultures. However, in cultures with LPDS, there was a significant effect of pravastatin at 20 μg/mL (Table 2).

In vivo *study*. Ten previously untreated patients with CLL received simvastatin, 40 mg daily orally for 12 wk. No patient experienced adverse reactions during medication. Some patients had slight changes in the degree of lymphadenopathy during simvastatin medication (Table 3). However, all these changes were compatible with "stable disease." Similarly, the hemoglobin level and platelet counts were stable during the medication in all 10 patients. The lymphocyte counts at baseline, after 12 wk of medication, and at 3 mon poststudy are given in Table 3. In most patients, there was a mild to moderate increase in lymphocyte counts during medication.

Before treatment, total plasma cholesterol ranged from 2.3 to 7.0 mmol/L (mean, 5.4 mmol/L). Mean reductions in total plasma and LDL cholesterol during simvastatin medication were 30% (range 9–46%) and 37% (range 16–63%), respectively (Table 3). Table 4 provides detailed lipid data at baseline and after 12 wk of therapy.

TABLE 4
Plasma Lipids in Patients with CLL at Baseline and Following 12 Wk of Therapy with Simvastatin (Zocord) 40 mg Daily[a]

		Plasma		LDL		HDL		VLDL	
No.	Week	Chol	TG	Chol.	TG	Chol.	TG	Chol.	TG
1	w0	2.3	1.2	1.7	0.4	0.3	0.2	0.3	0.6
	w12	2.1	0.8	1.1	0.2	0.6	0.2	0.4	0.4
2	w0	4.4	1.3	3.2	0.6	0.8	0.3	0.4	0.4
	w10	3.7	0.9	2.7	0.5	0.8	0.2	0.2	0.2
3	w0	5.5	2.1	3.9	0.7	0.9	0.3	0.7	1.1
	w12	4.1	1.2	2.7	0.5	1.0	0.3	0.4	0.4
4	w0	5.9	1.7	4.4	0.6	0.8	0.2	0.7	0.9
	w12	3.8	1.1	2.8	0.4	0.6	0.2	0.4	0.5
5	w0	6.3	2.5	4.5	0.5	0.7	0.3	1.1	1.7
	w12	4.0	1.4	2.7	0.3	0.8	0.2	0.5	0.9
6	w0	5.0	1.1	3.9	0.4	0.7	0.3	0.4	0.4
	w12	3.8	1.0	2.7	0.3	0.8	0.3	0.3	0.4
7	w0	6.6	1.7	4.9	0.5	0.7	0.2	1.0	1.0
	w12	4.4	1.1	3.4	0.3	0.8	0.3	0.2	0.5
8	w0	5.3	1.3	3.2	0.2	1.7	0.2	0.4	0.9
	w12	3.3	1.6	1.9	0.3	1.0	0.4	0.4	0.9
9	w0	7.0	1.4	5.9	0.6	0.8	0.3	0.3	0.5
	w12	4.3	0.6	3.2	0.3	0.9	0.1	0.2	0.2
10	w0	6.1	1.0	5.4	0.3	0.5	0.1	0.2	0.6
	w12	3.3	0.9	2.0	0.5	0.9	0.1	0.4	0.3

[a]All concentrations are in mmol/L. Chol, cholesterol; TG, triglycerides; w0, week 0 (baseline level); w12, week 12 (after 12 wk of drug treatment). One patient (no. 2) was sampled after 10 wk of therapy. HDL, high density lipoprotein; VLDL, very low density lipoprotein. See Table 3 for other abbreviations.

The mean high affinity degradation rate of ^{125}I-LDL in mononuclear cells, directly after isolation from blood, was 0.12 (range 0–0.57) ng × h^{-1} × 10^6 $cells^{-1}$ (n = 10). During simvastatin therapy, cells from patient no. 1 showed the highest absolute increase in the high affinity degradation rate of ^{125}I-LDL, whereas cells from three other patients (nos. 4, 5, 6) only showed minor increases (Fig. 1). Following 48 h of incubation in medium with 10% LPDS, the inducible LDL receptor activity increased mainly in cells from the same patients (Fig. 2). HMG–CoA reductase activity seemed to increase during simvastatin medication in three patients (nos. 4, 9, 10; Fig. 3) with the maximum levels reached at end of therapy (week 12). Patient no. 2 showed a late and marked increase from week 10 to week 28. He was not assayed at week 12.

During follow-up, four patients developed progressive disease. One patient (no. 1) with massive lymphadenopathy had recently acquired slight general symptoms at inclusion into study. These symptoms remained unchanged during simvastatin medication, and he was put on chlorambucil and prednisone immediately after withdrawal of simvastatin. Chlorambucil and prednisone also failed to improve his status, and he subsequently received intensive combined chemotherapy. Patient no. 6 developed blastic transformation in neck lymph nodes 3 mon after the withdrawal of simvastatin, and was put on chemotherapy. Patient no. 4 developed progressive lymphadenopathy and slowly but steadily increasing general symptoms, and responded well to chlorambucil given 8 mon after the end of the simvastatin therapy. A fourth patient (no. 7) developed progressive splenomegaly with slight anemia and thrombocytopenia, and was splenectomized a year after simvastatin therapy. Patients nos. 2 and 10 had fludarabine for progressive disease between 2 and 3 yr after stopping simvastatin.

DISCUSSION

We here show that the mitogen-induced thymidine uptake of cultured CLL cells is suppressed by the cholesterol synthesis inhibitors lovastatin, simvastatin, and pravastatin. Suppression by pravastatin was evident only when cells were cultured in medium with LPDS.

To detect suppression of cell proliferation, there must be a significant proliferation in the control culture. Since malig-

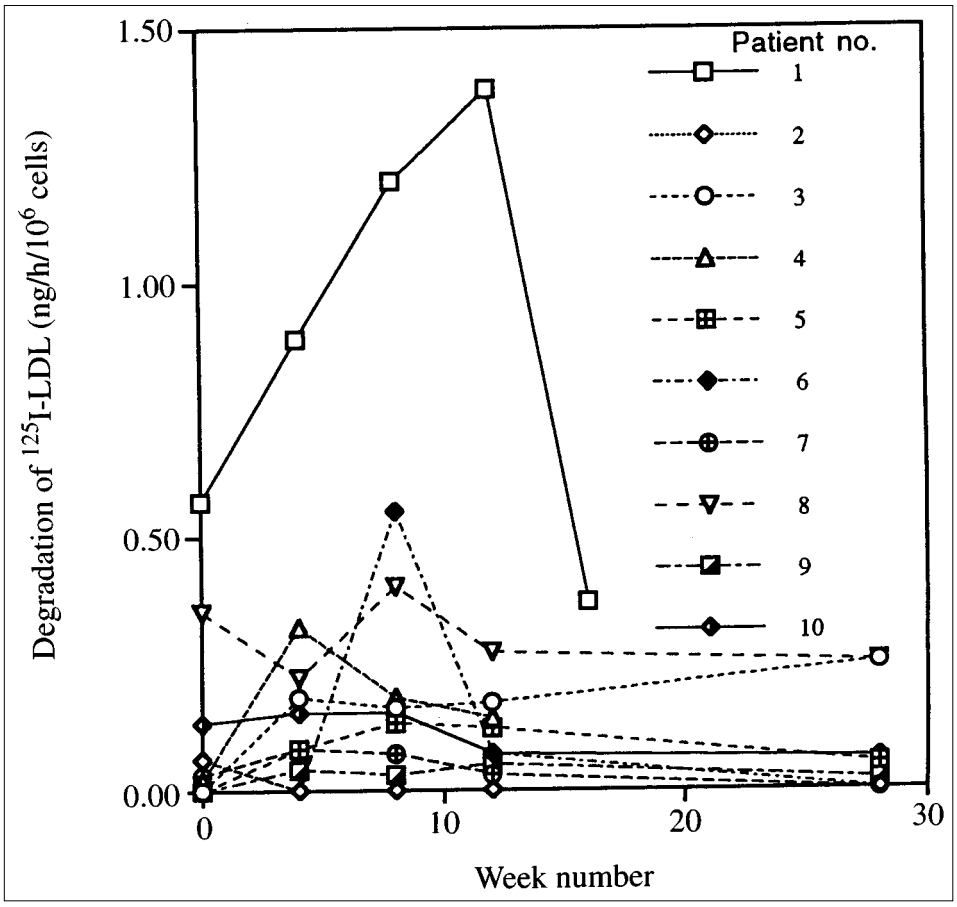

FIG. 1. High affinity degradation rate of ^{125}I-low density lipoprotein (LDL) in mononuclear blood cells from 10 patients with chronic lymphocytic leukemia directly after isolation from blood. Week 0 denotes the degradation value prior to medication. Simvastatin medication was from week 0 to week 12.

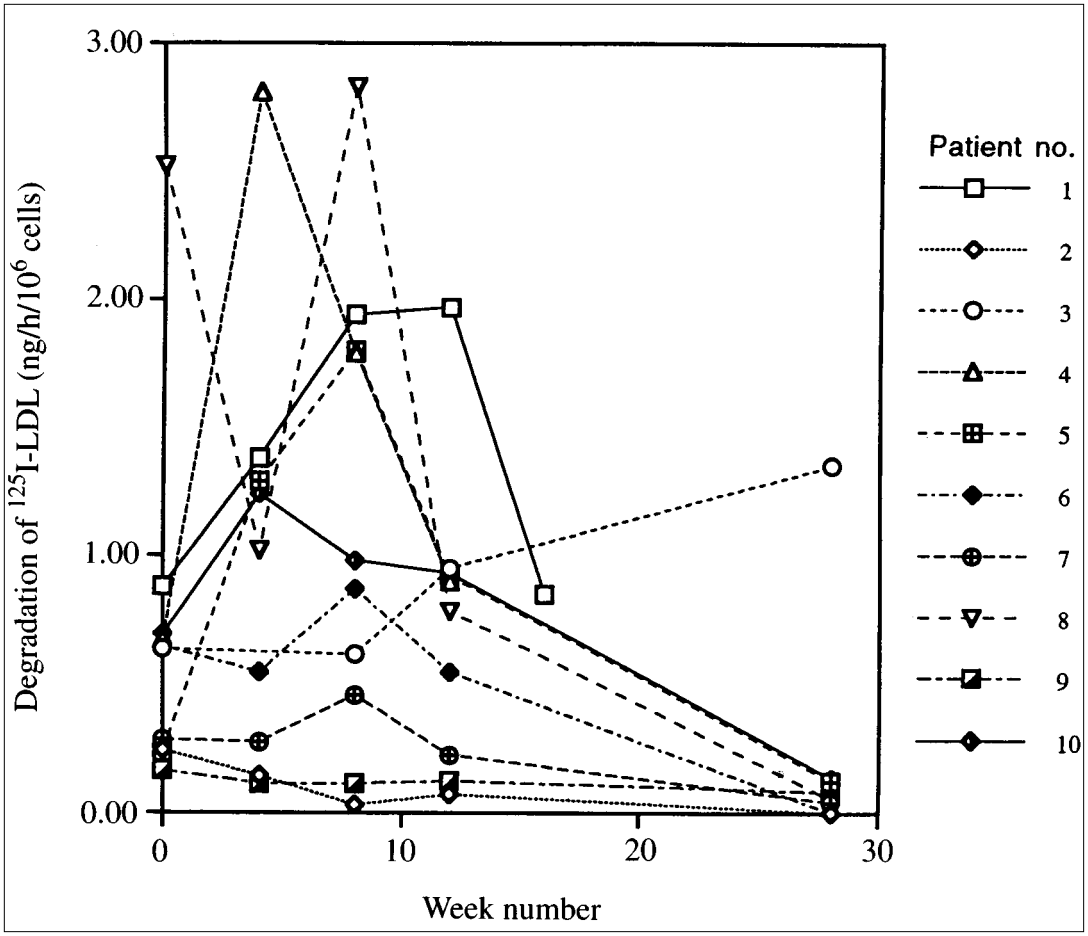

FIG. 2. High affinity degradation rate of ^{125}I-LDL in mononuclear blood cells from 10 patients with chronic lymphocytic leukemia following 48 h of incubation in medium containing 10% lipoprotein-deficient serum. Week 0 denotes the degradation value prior to medication. Simvastatin medication was from week 0 to week 12. See Figure 1 for abbreviation.

nant B-lymphocytes in general are nonproliferating *in vitro,* we have used a test system in which proliferation was induced by B-cell mitogens (13). Thymidine uptake in B-cell mitogen-stimulated CLL cell cultures reflects the proliferative activity of the malignant B-cells more than that of contaminating normal cells. This has previously been demonstrated by cytogenetic studies (14), by mitogen-induced change in CLL cell phenotype (15), and by the induction of clonal Ig-secretion in B-cell mitogen-stimulated CLL cell cultures (13,16). However, similar effects were also seen in spontaneously proliferating cells, such as bone marrow cells from CLL patients (data not shown). Thus, the drug effect is not likely to be directed against the action of the mitogen but rather against the cells' ability to proliferate.

We have previously found that CLL cells have a greatly reduced LDL receptor activity, both when freshly isolated and following incubation in medium with LPDS. The low LDL receptor activity in freshly isolated cells (mean 0.12 ng $\times$ h^{-1} $\times$ 10^6 cells^{-1}) in the current study confirms previous findings (5). For comparison, in mononuclear cells from healthy individuals, the mean high affinity degradation rates of ^{125}I-LDL were 0.35 (range 0.22–0.56, n = 18) (3) and 0.50 (range 0.25–0.83, n = 23) (17) ng $\times$ h^{-1} $\times$ 10^6 cells^{-1}, respectively. Since proliferating cells have a demand for cholesterol for their membrane synthesis, the reduced CLL cell proliferation in the presence of cholesterol synthesis inhibitors is probably due to reduced availability of cholesterol. Normal cells should be able to compensate for this lack of endogenous cholesterol synthesis by increasing their LDL receptor activity and take up more LDL cholesterol from blood, whereas CLL cells should be more severely affected. The finding in this study that simvastatin and lovastatin showed similar efficacy in cultures with standard serum as compared to LPDS is encouraging and supports that cholesterol in plasma does not counteract the antiproliferative effect of the drugs toward CLL cells. That the antiproliferative effects by cholesterol synthesis inhibitors in this study were mediated by inhibition of HMG–CoA reductase is supported by the facts that mevalonate has been shown to counteract inhibition of mitogen stimulated lymphocyte proliferation (18) and tumor cell growth (19) by lovastatin.

Lovastatin and simvastatin have few side effects, as

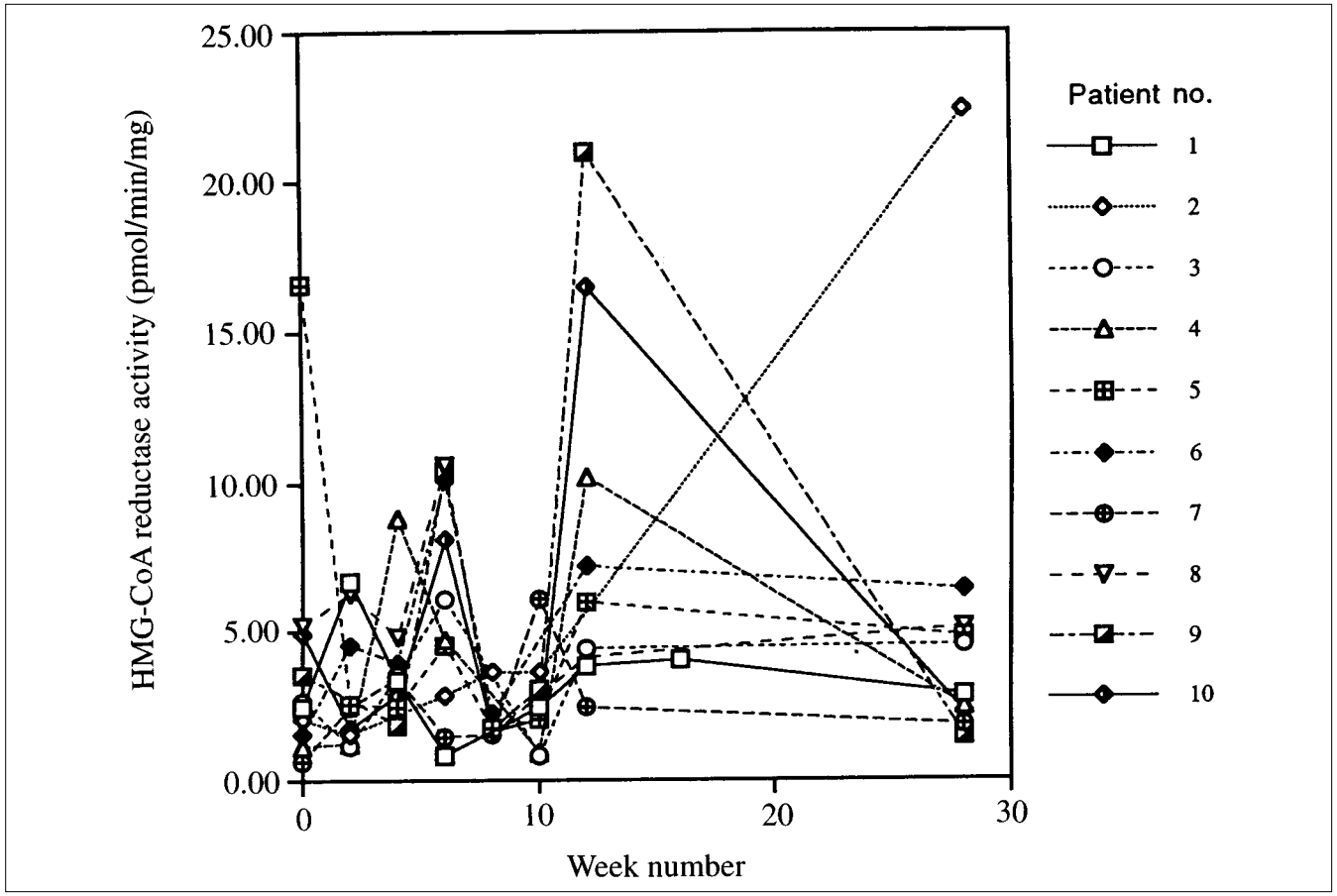

FIG. 3. 3-Hydroxy-3-methylglutaryl–CoA reductase activity in mononuclear blood cells from 10 patients with chronic lymphocytic leukemia directly after isolation from blood. Week 0 denotes the degradation value prior to medication. Simvastatin medication was from week 0 to week 12.

demonstrated in large-scale studies of treatment for hypercholesterolemia. Previous studies have shown that lovastatin suppressed murine neuroblastoma growth *in vivo* (20), and antitumoral effects have also been demonstrated with lovastatin in an animal tumor model of pancreatic adenocarcinoma (19). It has also been recently shown that simvastatin could selectively inhibit the growth of acute myelogenous leukemia cells *in vitro* (21). Interestingly, lovastatin has also been shown to block the activity of the oncogen *ras* (22,23) although *ras*-activation has not been shown to be involved in CLL pathogenesis (24). A phase I study, evaluating maximum tolerated dose of orally given lovastatin to cancer patients, recently has also been conducted (25).

We tested, in an open nonrandomized pilot study, the hypothesis that simvastatin could prevent progression of CLL. Thus, 10 CLL patients with a wide range of initial plasma cholesterol were given 40 mg simvastatin daily for 12 wk. Plasma cholesterol and LDL cholesterol were reduced to about two-thirds of initial values during treatment, which is not different from the expected result in a nonleukemic population (26). No significant change in tumor volume could be detected during the treatment period. None of the ten patients had evidence of progressive disease during simvastatin medication, and none became symptomatic. However, four of the patients developed progressive CLL disease upon cessation of therapy during the subsequent year.

CLL cells from some of the patients showed moderate to minor increases in LDL receptor and/or HMG–CoA reductase activities during medication. The mechanism behind this phenomenon can only be speculated upon. It is possible that this reflects a drug effect on the CLL cells per se, but we cannot rule out the possibility that this is an effect secondary to the reduced serum cholesterol levels. The fact that, during simvastatin medication, cells from some patients also had higher LDL receptor activity following incubation for 48 h in medium with LPDS does not necessarily imply that a higher maximum capacity was reached. It could, for example, be explained by a "kinetic advantage" owing to the fact that higher degradation values are reached more rapidly since cells from these patients also had elevated receptor activity directly after isolation from blood. Cells from some CLL patients may therefore have the capacity to circumvent cholesterol deprivation by upregulating the synthesis of HMG–CoA reductase and/or increase the expression of LDL receptors. It is noteworthy that patient no.1, who had an aggressive disease, had the most pronounced increase in LDL receptor activity during medication. This patient also had the lowest serum cholesterol level at baseline, highest LDL receptor activity at

baseline, and showed an increase in the white blood cell count during medication. A prospective analysis of such a compensatory capacity before patients are entered in any future trial with the goal to correlate with eventual treatment effects should be of interest.

It is known that the inhibitory effect of oral simvastatin on HMG–CoA reductase activity is very low in blood and peripheral tissues, due to extensive first-pass metabolism, high extraction by the liver, and high protein binding in plasma of drug and active metabolites (27). We are aware that an optimal dose was not chosen in the current pilot study. It is also possible that antitumoral effects develop more slowly and that the study has to be considerably prolonged. Thus, the hypothesis that simvastatin may prevent CLL cell proliferation *in vivo* cannot be rejected at this stage. A larger controlled study, preferably using higher doses of an intravenously administered HMG–CoA reductase inhibitor, should be of interest in CLL.

ACKNOWLEDGMENTS

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Differentiation of Human Promyelocytic Leukemia Cell Line HL60 by Microbial Extracellular Glycolipids

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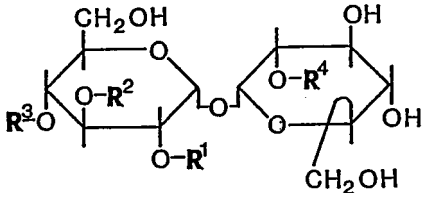
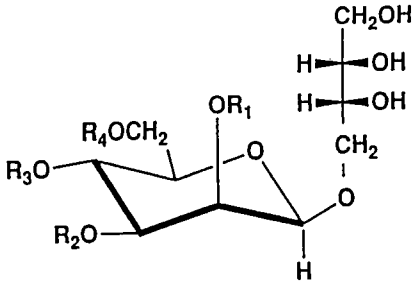
ABSTRACT: Microbial extracellular glycolipids, succinoyl trehalose lipid (STL), and mannosylerythritol lipid (MEL) inhibited the growth of a human promyelocytic leukemia cell line, HL60, and induced their morphological changes. The results of specific and nonspecific leukocyte esterase activities showed that STL induced monocytotic differentiation while MEL induced granulocytic differentiation. STL and MEL markedly increased common differentiation-associated characteristics in monocytes and granulocytes, such as nitroblue tetrazolium (NBT) reducing ability, expression of Fc receptors, and phagocytic activities in HL60 cells, respectively. Neither sugar moieties nor fatty acids in the free form, the individual components of STL and MEL, were effective at inducing the differentiation of HL60 cells. The induction of differentiation was not due to surface activities of STL and MEL on the basis of the complete ineffectiveness of the analogues tested. The composition of cell surface glycosphingolipids (GSL) changed such that the G_{M3}/LacCer ratio increased in STL-treated cells, whereas it decreased in MEL-treated cells. HL60 cells treated with STL and MEL exhibited a significant decrease in the activity of the intracellular phospholipid- and Ca^{2+}-dependent protein kinase (protein kinase C). Furthermore, the serine/threonine phosphorylations in intact HL60 cells were clearly inhibited by the presence of G_{M3} and MEL, but not by LacCer and STL. These results suggest that the differentiation-inducing activity of STL and MEL is not due to a simple detergent-like effect but due to a specific action on the plasma membrane. The inhibitory effect of STL on protein kinase activity was through increasing G_{M3}, but MEL had a direct inhibitory effect.

Lipids 32, 263–271 (1997).

Several species of microorganisms are known to produce compounds which have surface-active characteristics generally termed "biosurfactants." Biosurfactants are typical amphiphilic molecules, containing both a lipophilic and hydrophilic moiety. Succinoyl trehalose lipid (STL) (R^{1-4} = 2× succinoyl + 2× alkanoyl) (Scheme 1) and mannosylerythritol lipid (MEL) (R^{1-4} = 2× acetyl + 2× fatty acid) (Scheme 2) are glycolipid-type biosurfactants produced by *Rhodococcus erythropolis* SD-74 grown on *n*-hexadecane (1) and *Candida antarctica* T-34 on soybean oil (2), respectively. Under optimized culture conditions, they were produced in concentrations of about 40 g per liter of culture broth (3,4).



SCHEME 1



SCHEME 2

Recently, it is reported that gangliosides and glycosphingolipids (GSL), which are ubiquitous membrane components, modulate cell growth, adhesion, and transmembrane signaling, because of dramatic changes in GSL composition and metabolism observed during oncogenesis, differentiation, and oncogenic transformation (5). In the case of mammalian glycolipids, the lipid portion of the molecule is attached *via* an *O*-glycosidic linkage to the reducing end of the oligosaccharide moiety. On the other hand, in the case of STL or MEL, the lipid part of the molecule is attached *via* an ester linkage to the ring hydroxyl groups. Although many obvious differ-

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Abbreviations: GSL, glycosphingolipids; MEL, mannosylerythritol lipid; NAE, α-naphthyl acetate esterase; NBT, nitroblue tetrazolium; NCAE, naphthol AS-D-chloroacetate esterase; PBS, phosphate buffered saline; PKC, protein kinase C; STL, succinoyl trehalose lipid.

ences exist between mammalian glycolipid and STL or MEL, such structures as glycolipids are similar. Therefore we assessed whether STL and MEL have an ability to induce cell differentiation using human cell line, HL60. It is a human promyelocytic leukemia cell line (6) that has been induced to differentiate into mature granulocytes (7,8) and macrophage-like cells (6,9) and consists of stem-like cells that are bipotent with respect to myeloid or macrophage differentiation (10).

In this study, we found of the microbial extracellular glycolipids, STL induced monocytoic differentiation while MEL induced granulocytic differentiation of HL60 cells, and the two glycolipids increased common differentiation characteristics in monocytes and granulocytes. In order to assess the mechanisms of HL60 cell differentiation-induction by STL and MEL, we examined the effects of their components and surface activities on the induction of cell differentiation. Furthermore, we analyzed the changes of GSL on cell surface membrane, the PKC activities, and the serine/threonine phosphorylation patterns during cell differentiation by STL or MEL treatment. In this paper, we show the leukemia cell differentiation–induction by microbial extracellular glycolipids and discuss its mechanisms.

MATERIALS AND METHODS

Materials. Succinic acid, trehalose, SDS, and sorbitan monolaurate (Span 20) were purchased from Wako Pure Chemicals (Osaka, Japan). GSL GM3 and LacCer, and hexadecanoate and tetradecanoate were purchased from Funakoshi (Tokyo, Japan). Sucrose monocaprate (SE 10) was purchased from Dojin Laboratories (Kumamoto, Japan).

Preparation of STL and MEL. STL was prepared by the method of Uchida *et al.*(1). Seed cultures were prepared by inoculating loopfuls of cells of strain SD-74 grown on the slants into 300-mL Erlenmeyer flasks containing a seed culture medium (30 mL), followed by incubation at 30°C for 2 d on a rotary shaker. The seed cultures (4 mL) were transferred to 500-mL Erlenmeyer flasks containing 50 mL of media containing 10% (vol/vol) *n*-hexadecane, and were shaken at 30°C for 7 d on a rotary shaker ($200 \times g$). The culture broths were centrifuged at $10{,}000 \times g$ for 30 min. The aqueous layer was carefully siphoned, collected, and acidified with 6 N HCl to pH 3.0. The acidic exolipids were precipitated as voluminous gel mass. The gel mass was washed twice with water and mixed with hot methanol (300 mL). The mixture was washed three times with *n*-hexane to remove minor lipids and remaining *n*-hexadecane, then concentrated. The residue was dried over P_2O_5 under reduced pressure and used as the mixture of the main exolipids. The mixture of main exolipids was dissolved in ethyl acetate (200 mL) and kept in cold room. STL was mainly obtained as precipitates. After repeated recrystallization with ethyl acetate, STL was obtained as a colorless powder. The long fatty acid composition of STL was hexadecanoate (87%) and tetradecanoate (13%). Found: C, 61.21; H, 8.97%. Calcd. for $C_{52}H_{90}O_{19}$ (hexadecanoate): C, 61.27; H, 8.90%.

MEL was prepared by the method of Kitamoto *et al.* (2). Seed cultures were prepared by inoculating cells grown on slants into 300-mL Erlenmeyer flasks containing 30 mL of a seed culture medium, followed by incubation at 30°C for 2 d on a rotary shaker ($200 \times g$). The seed cultures (2 mL) were transferred to twenty 500-mL Erlenmeyer flasks containing 50 mL of a basal fermentation medium, followed by incubation at 30°C for 7 d on a rotary shaker. The culture broths were combined acidified with 6 N HCl to pH 3.0, and centrifuged at $2{,}850 \times g$ for 30 min. The precipitate was washed twice with water by centrifugation, and the heavy oil was extracted twice with methanol (300 mL). The combined extracts were concentrated (100 mL) then washed twice with two volumes of *n*-hexane to remove the remaining oil and free fatty acids. Chloroform (200 mL) and water (100 mL) were added to the washed concentrate, and the mixture was shaken. The lower chloroform layer was evaporated to dryness after dehydration with Na_2SO_4. Removal of the solvent left a syrup, which was used as the mixture of biosurfactants in the following experiments. The mixture of biosurfactants was dissolved in chloroform and placed on a column (3×40 cm) of silica gel (Wakogel C-200; Wako). The column was first washed with 400 mL of chloroform/eyhyl acetate (4:1). The component (MEL) was eluted with 800 mL of chloroform/acetone (7:3). The long fatty acid composition of MEL consists octanoate (17.5%), decanoate (10.1%), and tetradecanoate (1.1%). Found: C, 59.95; H, 8.60%. Calcd. for $C_{34}H_{60}O_{13}$ (2 acetyl, 2 decanoyl): C, 60.33; H, 8.94%.

MEL and the long-chain fatty acid of MEL were obtained by deacylation. After acidification of the reaction mixture, the long-fatty acids were extracted with *n*-hexane and evaporated. The aqueous layer containing MEL was desalted and concentrated.

Cells and cell culture. HL60 cell line was obtained from Riken Cell Bank (Tsukuba, Ibaraki, Japan). The cells were cultured in a serum-free ERDF (Kyokuto Pharmaceutical Kogyo Co., Tokyo, Japan)–ITES medium at 37°C in humidified 5% CO_2/95% air. ITES is 5 μg/mL insulin, 10 μg/mL iron-free human transferrin, 25 μM ethanolamine, and 25 nM selenite (RD-1; Kyokuto Pharmaceutical Kogyo Co.). Growth of HL60 cells is known to be insulin-dependent (11).

The cells were counted using a hemacytometer, and the viability was estimated by the Trypan blue dye exclusion method. Test samples, such as STL, MEL, their components, and other surfactants dissolved in phosphate buffered saline (PBS) were sonicated and sterilized by passage through a MILLEX-GV 0.22 μm filter unit (Millipore Products Division, Bedford, MA). They were added to the culture medium at the desired sample concentration. The final PBS concentration was kept at 0.1%. For control, the cultured cells were added with only PBS (without sample treatment).

Determination of cell differentiation. HL60 cells were cultured with STL or MEL for 5 d. Cells that adhered to the culture vessels were removed with a Nunc 179693 plastic cell scraper (Nunc Inter Med., Roskilde, Denmark) and both adhered and nonadhered cells were investigated.

For cytochemical assessment of cell differentiation, specific and nonspecific leukocyte esterase activities (12) were determined cytochemically by the esterase double-staining method for α-naphthyl acetate esterase (NAE) and naphthol AS-D-chloroacetate esterase (NCAE) using reagents for determination of esterase (Sigma Chemical Co., St. Louis, MO). Specific and nonspecific esterase activities were examined on days 2 and 5.

To assess the differentiation of HL60, common differentiation-associated characteristics in monocytes and granulocytes, such as nitroblue tetrazolium (NBT) reducing ability, expression of Fc receptors, and phagocytic activities were investigated.

The NBT-reducing ability was determined by the modified method of Takeda *et al*. (13). Briefly, after treatment with STL or MEL for 2 d, viable cells (2×10^5) were washed once with ERDF medium and were suspended in 2 mL of ERDF medium containing 5% fetal bovine serum, 0.1% NBT dye in a 12-well plate for 2 h at 37°C in a 5% CO_2 incubator. The percentage of cells containing blue–black formazan deposits was determined by counting at least 200 cells under microscope for each experimental point.

The expression of Fc receptors on the cell surface of HL60 was assayed by EA rosette formation. HL60 cells (2×10^5) were washed with ERDF medium after being treated with STL or MEL for 3 d, and suspended in 2 mL of 0.9% NaCl, then incubated with suspension of bovine erythrocytes (5×10^6/mL) sensitized with rabbit IgG antibodies (Funakoshi) at 22°C for 60 min. Cells with more than three attached erythrocytes were counted as positive. At least 200 cells were examined for each experimental point (14). The phagocytic activity of HL60 cells was measured by counting the percentage of the cells that phagocytosed yeast particles.

The yeast–phagocytic activity was investigated with opsonized yeast. Heat-killed yeast particles (*Saccharomyces cerevisiae:* Wako Pure Chemical Industries Ltd.) were incubated with three times volumes of mouse serum (Funakoshi) for 30 min at 37°C and washed with Hanks balanced salt solution. After treatment with STL or MEL for 3 d, the cell (2.0×10^5) pellet was mixed with 5.0×10^6 of opsonized yeast particles suspended in 2 mL of Hank's balanced salt solution and incubated for 60 min at 37°C. Cells were washed again and 2 mL of 0.01% fuchsin solution was added to the cell pellet. The cells containing yeast particles were scored as phagocytosis positive, and at least 200 cells were counted for each experimental point (13).

Extraction and determination of GSL composition. GSL from the cell were extracted and determined by the modified method of Tsuruoka *et al.* (15). Harvested 10^7 cells were washed with 1 mL of Ca^{2+}- and Mg^{2+}-free PBS. After centrifugation, the packed cells were combined with 0.5 mL of isopropyl alcohol/hexane/water (55:25:20, by vol) and homogenized by sonication. The pellet was extracted twice with the same solvent and finally extracted three times with 0.5 mL of chloroform/methanol (2:1). All the extracts were evaporated to dryness under N_2 stream. The residue was dissolved in 0.5 mL of 0.1M KCl, passed through a Seppak Plus C_{18} Cartridge (Millipore Corporation), and washed with 2 mL of 0.1 M KCl followed by 5 mL of distilled water and eluted with 2 mL of CM (2:1). The final elute was evaporated to dryness, and the residue was dissolved in 200 µL of CMW (2:1:0.05). The sample was analyzed on a high-performance thin-layer chromatography (HPTLC) plate (HPTLC-Fertigplatten, Kieselgel 60; Merck AG, Darmstadt, Germany) and developed with CMW (50:40:10). The GSL were detected by 0.2% orcinol in 2 M sulfuric acid. The relative amounts of the components on the chromatograms were estimated with a densitometer (TLC Scanner CS-920; Shimadzu, Tokyo, Japan).

Kinase assay of PKC. After treatment with STL or MEL for 24 h, 10^7 cells were washed twice with cold Ca^{2+}- and Mg^{2+}-free PBS. After centrifugation at $3350 \times g$ for 2 min, the packed cells were homogenized with 200 µL of homogenized solution (20 mM Tris-HCL, pH7.5, 2 mM EDTA, 0.25M sucrose, 50 mM 2-mercaptoethanol, 0.1 mg/mL leupeptin, 2 mM PMSF) by sonication with 3 strokes at 4°C. The sonicate was centrifuged at $8500 \times g$ for 25 min at 4°C and obtained about 200 µL of cytosol (16). The cytosol was assayed for nonradioactive detection of protein kinase C (PKC) by PepTag™ Assay (Promega Corporation, WI).

Western analysis of the cells treated with glycolipids. HL60 (2×10^6 cells) were incubated with 50 µM of GM3, 50 µM of LacCer, 5 µM of STL and 10 µM of MEL. The reactions were stopped by adding 100 µl of cell suspension to the same volume of 2× Laemmli's sample buffer (1× is 62.5 mM Tris-HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 8.5% glycerol, 2.5 mM orthovanadate, 10 mM paranitrophenylphosphate, 12 µg/mL leupeptin, 12 µg/mL aprotinin, 1.25 mM PMSF, 0.025% bromophenol blue) preheated at 95°C. The samples were boiled for 5 min to completely denature the proteins and were then loaded onto 12.5% SDS-PAGE (17).

Electrophoretic transfer cells were used to transfer proteins from the gels to Immobilon PDVF membranes (Millipore Corporation). Nonspecific sites were blocked using 1X Block Ace (Dainihon Pharmaceutical Industry, Osaka, Japan) for 2 h at room temperature. The anti-phosphoserine (mono, X80; Daiichi-Kagaku, Tokyo, Japan) and phosphothreonine (mono, X160) antibodies were then incubated with the membranes for 2 h at room temperature in fresh blocking solution. The membranes were washed three times at room temperature in TBS-Tween and then incubated with horseradish peroxidase-labeled sheep anti-mouse IgG (Cosmo Bio, Tokyo, Japan) for 1 h at room temperature at a final dilution of 1:1500 in 10X Block Ace. The membranes were washed three times with TBS-Tween and the phosphopeptide bands were revealed using Konica Immunostein HRP-1000 (Konica, Tokyo, Japan).

RESULTS

Effect of STL and MEL on HL60 cell growth and cell differentiation. The effects of STL and MEL on the growth of HL60 were examined. During the 5 d of culture, STL at a con-

centration of 5.0 μM almost inhibited the growth of HL60. On day 5, the cell numbers which were treated with 2.5 μM of STL were less than 50% as compared to control. It was toxic to the HL60 cells at higher concentrations (>15 μM) (Fig. 1A). MEL at a concentration of 10.0 μM completely inhibited the growth of HL60 during the 5 d of culture, and was toxic at higher concentrations (>25 μM). MEL (5.0 μM) also inhibited the growth of HL60 cells (Fig. 1B). On the second day of cultivation, morphological changes of HL60 were observed in both STL- and MEL-treated cultures. HL60 cells treated with STL aggregated and were characterized by an increase in cell size, and the nuclear/cytoplasmic ratio was decreased (Fig. 2B). HL60 cells treated with MEL exhibited drastic morphological changes, and adhered to the bottom of the flask (Fig. 2C).

When HL60 cells were cultured with STL, the number of NAE-positive cells remarkably increased in a time-dependent manner. STL (5.0 μM) induced more than 65% NAE-positive

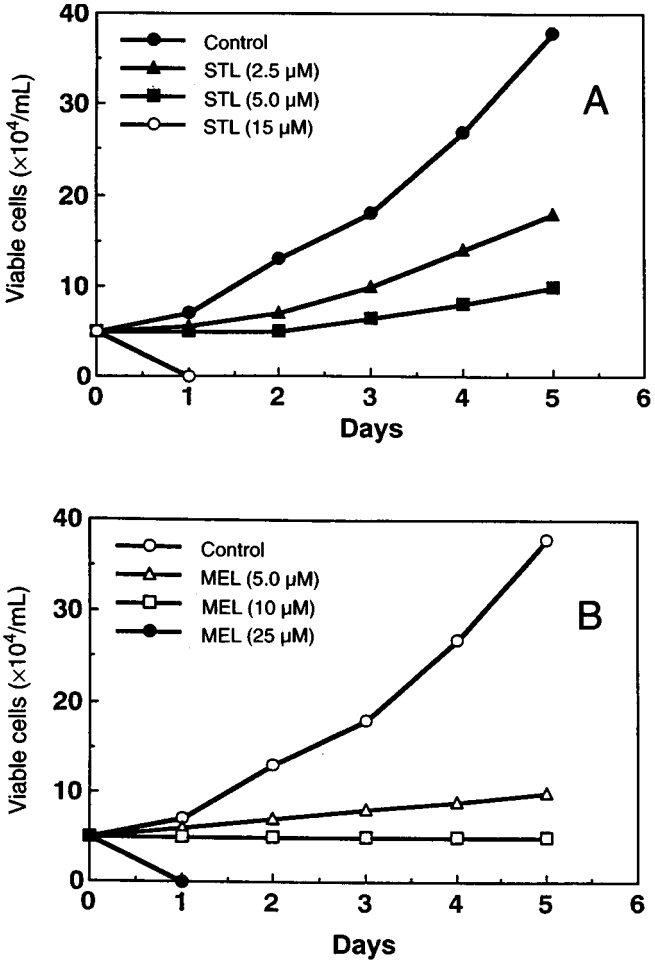

FIG. 1. Effect of succinoyl trehalose lipid (STL) (A) or mannosylerythritol lipid (MEL) (B) on growth of HL60 HL60 cells were seeded at an initial concentration of 5.0×10^4 cells per mL and grown in the ERDF–ITES medium with or without STL or MEL. Cells were counted with a hemacytometer, and viability was estimated by Trypan blue dye exclusion. Each data point represents the mean of three measurements. Standard deviation was <10%.

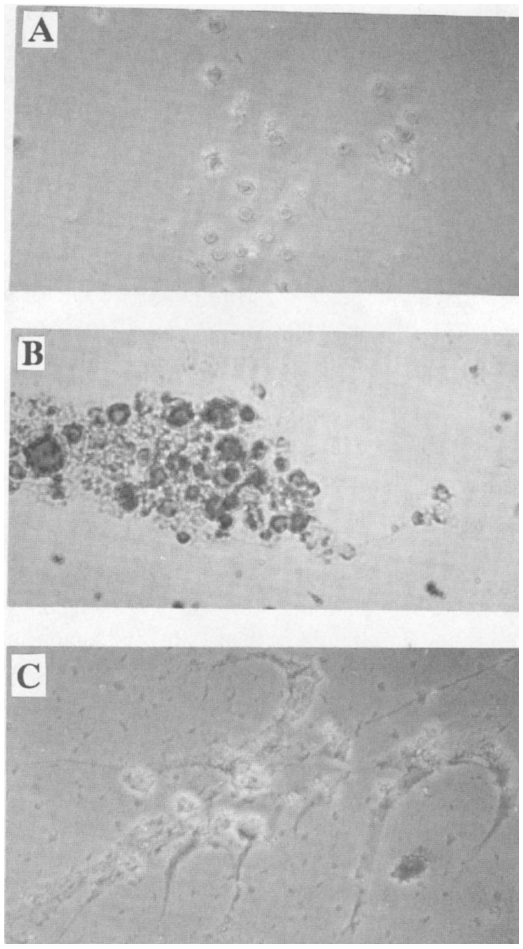

FIG. 2. Specific and nonspecific esterase staining of HL60 cells cultured in the ERDF–ITES medium with 5 μM of STL or 10 μM of MEL for 2 d. Specific and nonspecific leukocyte esterase activity was determined cytochemically by the esterase double-staining method as described in the Materials and Methods section Untreated HL60 cells (A), 5 μM of STL-treated HL60 cells (B) and 10 μM of MEL-treated HL60 cells (C). See Figure 1 for abbreviations.

cells on day 2, and on day 5, almost 80% cells were stained-NAE-positive. The cells treated with 2.5 μM of STL were also observed to be 55% NAE-positive (Fig. 3A). This esterase activity indicated the specificity for the monocytic lineage. On the other hand, when treated with MEL, NCAE-positive cells increased in a time-dependent manner. MEL (10.0 μM) induced almost 80% NCAE-positive cells on day 2, and it reached 90% on day 5 (Fig. 3-B). This esterase activity indicated the specificity for the granulocytic lineage.

From these results, HL60 cells treated with STL or MEL were assumed to be a manifestation of the differentiation of these cells to monocytes or granulocytes. To confirm this assumption, the following differentiation-associated characteristics of HL60 cells were investigated: NBT-reducing ability, Fc receptor expression and phagocytic activity.

NBT-reducing ability is often used as a marker for neutrophile, monocyte, macrophage, and lymphocyte which are known to exhibit toxicity on the numerous pathogenic bacteria

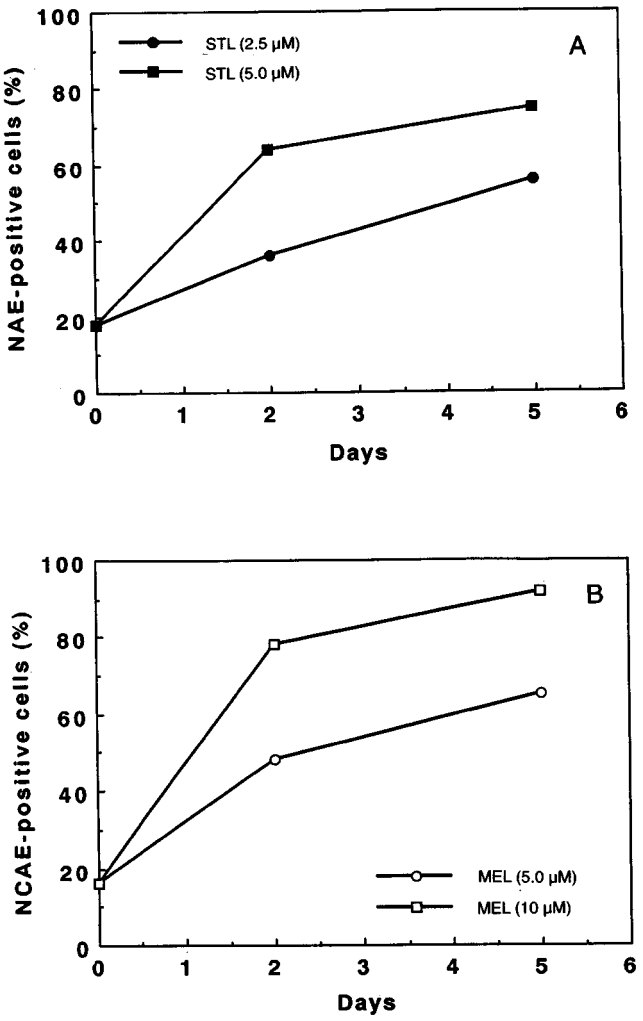

FIG. 3. α-Naphthyl acetate esterase (NAE) activity of STL-treated HL60 cells (A) and Naphthol AS-D-chloroacetate esterase (NCAE) activity of MEL-treated cells (B). HL60 cells were cultured in the ERDF–ITES medium with 2.5, 5.0 μM of STL or 5.0, 10.0 μM of MEL for 5 d. NAE and NCAE activities were determined by the method as described in the Materials and Methods section. The number of NAE- or NCAE-positive cells were determined on day 2 and day 5 of culture. Values are mean of three determinations. Standard deviation was <10%; NBT, nitroblue tetrazolium. See Figure 1 for other abbreviations.

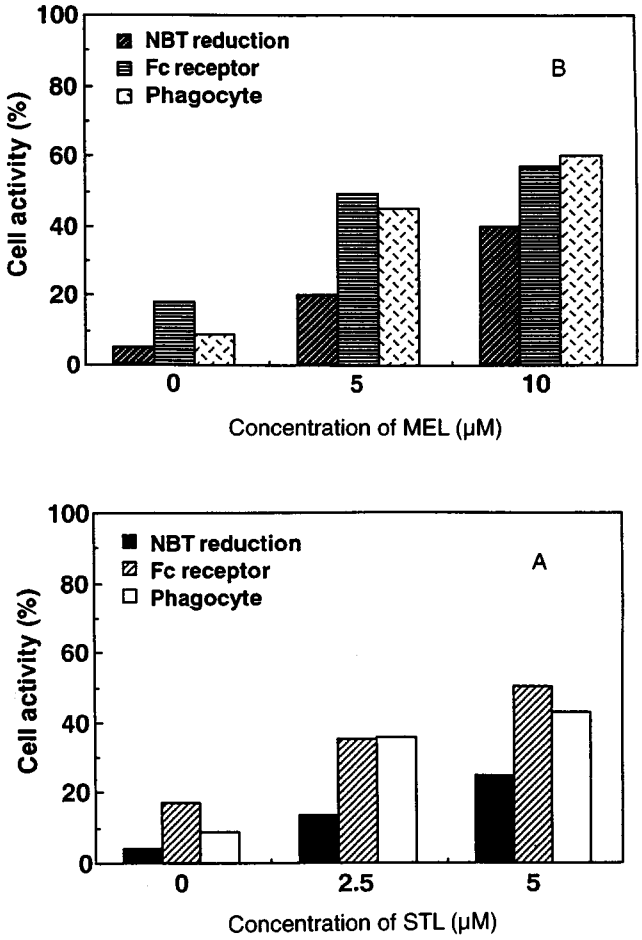

FIG. 4. Differentiation-associated characteristic properties of STL (A) or MEL (B) treated cells. NBT-reducing activity was determined by colorimetric assay. Assay of the appearance of Fc receptors was performed by standard techniques for erythrocyte–antibody rosette formation. Phagocytic activity is expressed as the percentage of cells ingesting yeast particles. They were described in the Materials and Methods section. Values are mean of three determinations. Standard deviation was <10%. See Figures 1 and 2 for abbreviations.

or cancer cells. This toxic response is demonstrated by their ability to reduce the water-soluble nitroblue tetrazolium dye to insoluble intracellular blue–black formazan due to O_2^- and H_2O_2 released upon activation. On the other hand, the process of phagocytosis by leukocyte involves two steps. The first step is bacterial recognition by leukocyte through bacterial surface immunoglobulin molecule (Fc). This step requires Fc receptor expression on the cell surface. The second step is the uptake of the bacteria into the leukocyte.

As shown in Figure 4A and B, both STL and MEL, respectively, induced the occurrence of NBT reducing ability, expression of Fc receptors, and phagocytic activities in HL60 cells significantly. The differentiation-associated characteristics of MEL-treated cells were above those of STL-treated cells. Especially, treatment of HL60 cells with 10 μM of MEL resulted in a remarkable increase in their Fc receptor expression and phagocytic activity reaching over 60%.

Effect of STL and MEL components and their surface activity. To assess the effect of components of glycolipids, the various components of the STL molecule, namely, succinic acid, trehalose and fatty acids as well as those of MEL such as mannosylerythritol and fatty acid, were added to HL60 cell cultivation separately, and the leukocyte esterase activity was examined. The growth of the cells treated with various components was not inhibited, and no morphological changes were detected. Each component of STL molecule, succinic acid, trehalose, the mixture of hexadecanoate and tetradecanoate as well as that of MEL such as mannosylerythritol and long-chain fatty acid had no effectiveness on the differentiation of HL60 cells in both cases of separate and simultaneous addition (Fig. 5). It was apparent that the ester molecule itself was responsible for the activity, since neither sugar

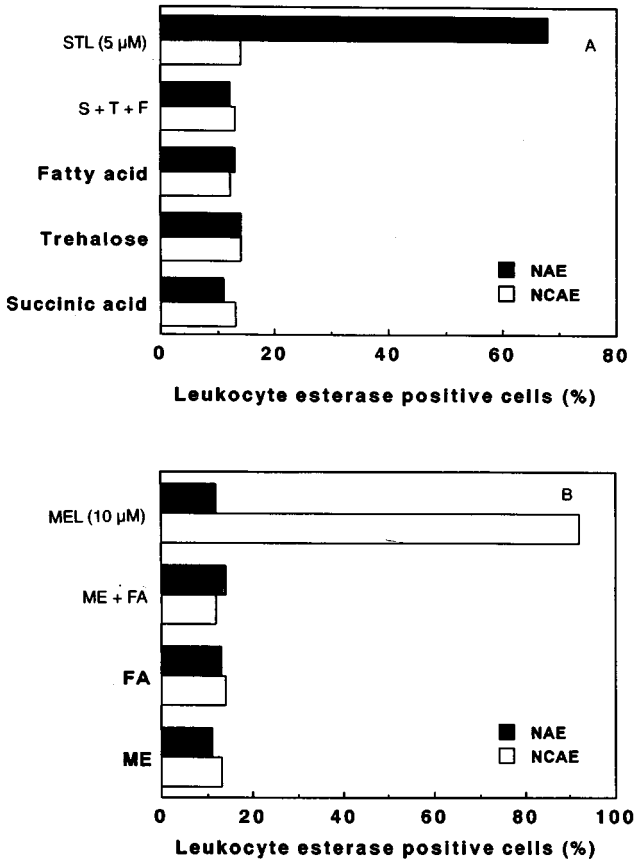

FIG. 5. Effect of components of STL molecule, succinic acid, trehalose, and fatty acids (A) and MEL molecule, mannosylerythritol (ME) and fatty acids (FA) (B) on differentiation–induction of HL60 cells. They were added to HL60 cell cultivation separately and leukocyte esterase activity examined. S + T + F means succinic acid, trehalose and fatty acid were together added to the culture medium. Values are mean of three determinations. Standard deviation was <10%. See Figure 1 for abbreviations.

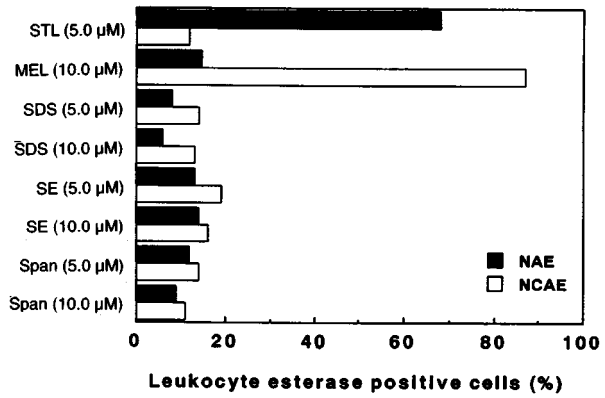

FIG. 6. Effect of analogues of STL and MEL on differentiation–induction of HL60 cells. 5.0 or 10.0 μM of STL or MEL and their analogues, SDS, sucrose monocaprate (SE 10) and sorbitan monolaurate (Span 20) were added to HL60 cell cultivation separately, and leukocyte esterase activities were examined. Values are mean of three determinations. Standard deviation was <10%. See Figures 1 and 5 for abbreviations.

moieties nor fatty acids of STL and MEL in the free form proved to be effective in inducing differentiation of cells.

On the other hand, it is reported that STL and MEL can act as anionic surfactant and nonionic surfactant with detergent properties, respectively (18,19). To confirm whether or not the ability of inducing differentiation of HL60 cells could be attributed to surface activities of these biosurfactants, we used analogues of STL and MEL. These were SDS (which is a typical anionic surfactant), sucrose monocaprate SE 10, and sorbitan monolaurate Span 20 (the last two being nonionic glycolipid-type synthetic surfactants). The results showed that surface activity had no effect on the induction of differentiation on the basis of the complete ineffectiveness of the samples tested (Fig. 6). From these results, we supposed that the differentiation-inducing activity of STL and MEL is not due to a simple detergent-like effect but due to a specific action on the plasma membrane.

GSL composition of STL- or MEL-treated HL60 cells. To confirm whether STL and MEL have specific action on the plasma membrane, the changes of GSL on cell surface membrane during cell differentiation were analyzed. The GSL pattern of non-treated and STL- or MEL-treated cells are shown in Figure 7. The major GSL of HL60 cells were identified as LacCer, Gb3Cer, GM3, and GM1 ganglioside by its TLC mobility, and the quantities of GSL in both nontreated and glycolipid-treated cells were shown in Table 1. GM3 ganglioside was increased in STL treated cells whereas LacCer was increased in MEL-treated cells compared to nontreated HL60 cells. The relative GM3/LacCer ratio of nontreated, STL-treated, and MEL-treated cells was 1.17, 2.45, and 0.30 on day 3, respectively.

Inhibition of kinase activity of PKC. The PepTag™ Assay utilizes brightly colored, fluorescent peptide substrates that are highly specific for PKC. Phosphorylation by PKC of their specific substrate alters the peptide's net charge from +1 to –1. This change in the net charge of the substrate allows the

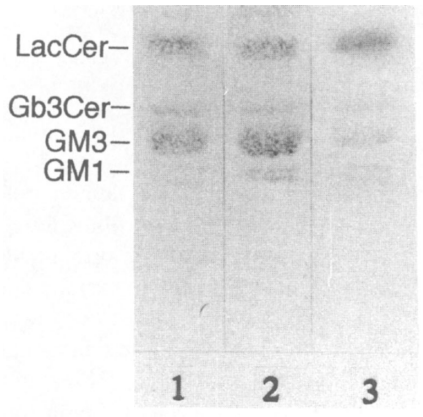

FIG. 7. High-performance thin-layer chromatography (HPTLC) pattern of neutral glycosphingolipids and ganglioside fraction of HL60 cells treated with or without STL and MEL. Samples were prepared as described in the Materials and Methods section. Each fraction was dissolved in 200 μL of CM (2:1), and 5 μL aliquots were spotted on HPTLC plates and developed with CMW (50:40:10). Lane 1, nontreated HL60 cells; Lane 2, STL-treated HL60 cells; Lane 3, MEL-treated HL60 cells. See Figure 1 for other abbreviations.

TABLE 1
Quantities of GSL in HL60 Treated with STL and MEL

	Chemical quantity[a] of			
	LacCer	Gb3Cer	GM3	GM1
Control	18.1	7.3	21.3	6.3
STL	24.1	7.4	59.1	7.3
MEL	37.8	5.3	11.5	3.3

[a]μg Equivalent to standard LacCer per 1 mL of packed cells. GSL, glycosphingolipid; STL, succinoyl trehalose lipid; MEL, mannosylerythritol lipid.

phosphorylated and unphosphorylated versions of the substrate to be rapidly separated on an agarose gel at neutral pH. The phosphorylated species migrates toward the positive electrode while the unphosphorylated substrate migrates toward the negative electrode.

On the control cells, phosphorylated peptide migrated toward the anode (+), while nonphosphorylated peptide migrated toward the cathode (–). On the other hand, on both cells which were treated with 5.0 μM of STL and 10.0 μM of MEL, phosphorylated peptide migrations were not detected (Fig. 8). These results showed that both STL and MEL at the concentration which induced differentiation of HL60 cells inhibited PKC activity in intact cells.

Comparison of the protein phosphorylation pattern. HL60 cells were treated with various glycolipids, and the resulting protein phosphorylation monitored. The results of threonine phosphorylation pattern after treatment of 3 h are shown in Figure 9. An examination of these data indicates that the threonine phosphorylation of a 30 kDa protein was observed in nontreated cells, and 50 μM of LacCer clearly enhanced the level of threonine phosphorylation. On the other hand, the phosphorylation of a 30 kDa protein was clearly inhibited by the presence of 50 μM of GM3 and 10 μM of MEL, but was only marginally inhibited by 5 μM of STL. After treatment with STL for 24 h, the phosphorylation of a 30 kDa protein was not observed (data not shown). With the use of the serine phosphorylation antibody, similar patterns were observed after treatment of both 3 h and 24 h (data not shown).

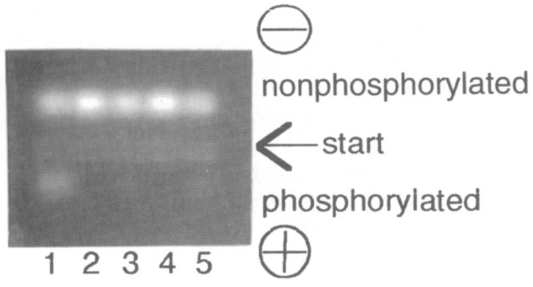

FIG. 8. Detection of protein kinase C. Samples were prepared as described in the Materials and Methods section. The samples were loaded on a 0.8% agarose gel and run at 100 V for 15 min. Phosphorylated peptide migrated toward the anode (+), while nonphosphorylated peptide migrated toward the cathode (–). The gel was photographed on a transilluminator. Lane 1, nontreated HL60 cells; Lane 2, 5.0 μM of STL-treated HL60 cells; Lane 3, 2.5 μM of STL-treated HL60 cells; Lane 4, 10.0 μM of MEL-treated HL60 cells; Lane 5, 5.0 μM of MEL-treated HL60 cells. See Figure 1 for abbreviations.

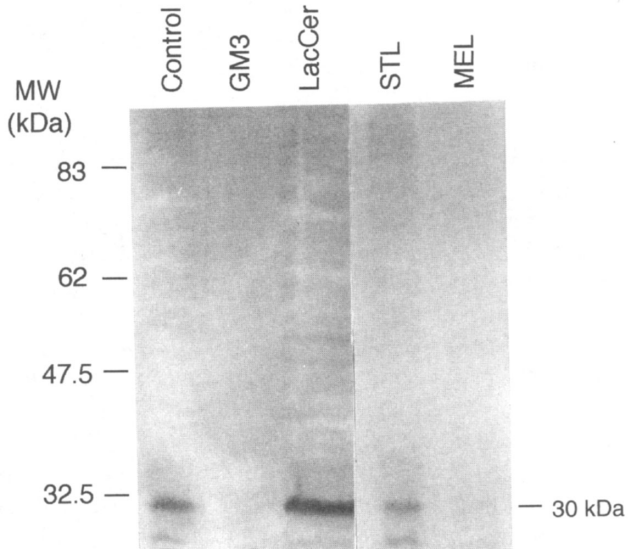

FIG. 9. Threonine phosphorylation pattern in non-, GM3, LacCer, STL, and MEL-treated HL60 cells for 3 h. Immunoblotting and revelation of phosphoserine and phosphothreonine were conducted, as described in the Materials and Methods section. See Figure 1 for abbreviations; MW, molecular weight.

DISCUSSION

In this paper, we have shown the differentiation–induction of HL60 cells by microbial extracellular glycolipids, STL and MEL. STL and MEL inhibited the insulin-dependent growth of HL60 cells and were also found enhancing the common differentiation-associated characteristics in monocyte and granulocyte, significantly. The cytologic demonstration of specific and nonspecific leukocyte esterase showed that STL induced monocytoic differentiation while MEL induced granulocytic differentiation. To investigate the mechanisms of their function on HL60 cells, the individual components of STL and MEL were examined, and neither sugar moieties nor fatty acids in the free form proved to be effective in inducing differentiation of cells. The induction of differentiation was not due to surface activities of STL and MEL on the basis of the complete ineffectiveness of the analogues tested. From these results, we supposed that the differentiation-inducing activity of STL and MEL is not due to a simple detergent-like effect but due to a specific action on the plasma membrane. Actually the GSL compositions of cell surface membrane were changed so that GM3 ganglioside increased after being treated with STL, whereas LacCer was increased after being treated with MEL compared to nontreated cells. GM3 and LacCer are components of cell surface membrane GSL, and also main components of monocyte–macrophages and granurocytes, respectively (20). Furthermore, both STL and MEL at the concentration which induced differentiation of HL60 cells exhibited a significant decrease of PKC activity. PKC comprises a family of serine/threonine protein kinases implicated in the cell regulation, differentiation, and proliferation (21,22). Inhibitors of PKC are expected to be antitumor agents because the tumor-

promoting phorbol esters are known to activate PKC directly (23,24). To confirm whether STL and MEL regulated protein kinases in HL60 cells directly or indirectly through inducing GSL such as GM3 and LacCer, the serine/threonine phosphorylation patterns were examined by the western analysis. The results showed that the serine/threonine phosphorylation of a 30 kDa protein which was observed in nontreated cells was clearly inhibited by the presence of 50 μM of GM3 and 10 μM of MEL, but was only marginally inhibited by 5 μM of STL in 3 h. After treatment with STL for 24 h, the serine/threonine phosphorylation of a 30 kDa protein was not observed. These results suggested that both STL and MEL may have interacted on the cell membrane, changed the GSL composition on the cell surface, and downregulated the serine/threonine kinase activities, then induced differentiation of HL60 cells, but the effects of STL or MEL were different. It was very likely that ganglioside GM3 that was specifically induced after STL treatment might be actually inhibiting the serine/threonine kinase, but MEL had direct inhibitory effect on it. Polysialogangliosides, lysoganglioside, and GM3 are reported to decrease PKC activity of HL60, brain, and A431, respectively (28). The critical micelle concentration of STL and MEL are 960 and 2.7 μM, respectively (17,18). As the effective concentration of STL (5 μM) is far less than the critical micelle concentration, while that of MEL (10 μM) is above CMC, MEL is assumed to form micelles, which could interact more on the cell membrane than STL.

It has been reported that certain gangliosides were found to induce differentiation of hepatopoietic cells upon exogenous addition to culture media (25–27). Nojiri *et al*. (28,29) reported that ganglio-series ganglio GM3 induced monocytoid differentiation, while neolacto-series ganglioside induced granulocytic differentiation of HL60. HL60 cells, which are bipotent with respect to the myeloid or monocytic pathway of differentiation, exhibited distinct quantitative changes in the GSL composition, depending not only on the differentiation stage but also on the differentiation direction. A remarkable increase in ganglio-series ganglioside GM3 with a concomitant decrease in lacto-series gangliosides is observed in the process of macrophage-like cell differentiation.

Concerning signal transduction of GSL, specific types of ganglioside are reported to inhibit receptor-associated tyrosine kinase activity, and therefore, gangliosides in the lipid bilayer surrounding the receptors are considered to have an important role in modulation of cell proliferation through direct or indirect interaction with receptor-associated tyrosine kinase (30–34).

Many obvious differences exist between mammalian glycolipids and STL or MEL, but they have similarities in both structures and functions. Further studies on the exact mechanism, linear signal transduction of differentiation–induction by microbial extracellular glycolipids are needed.

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Altered Phospholipid Metabolism in Sodium Butyrate-Induced Differentiation of C6 Glioma Cells

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ABSTRACT: We examined the changes in phospholipid metabolisms in sodium butyrate-treated C6 glioma cells. Treatment of 2.5 mM sodium butyrate for 24 h induced an increase in the activity of glutamine synthetase, suggesting that these cells were under differentiation. Similar treatment was associated with (i) increased arachidonic acid incorporation into phosphatidylcholine, and (ii) decreased arachidonic acid incorporation into phosphatidylinositol and (iii) phosphatidylethanolamine. These effects were subsequently investigated by examining the acylation process, *de novo* biosynthesis, and the agonist-stimulated phosphoinositides hydrolysis in these cells. Our results indicated that sodium butyrate stimulated the acylation of arachidonic acid into lysophosphatidylcholine, lysophosphatidylethanolamine, and lysophosphatidylinositol. The glycerol incorporation into these lipids was not affected, but the inositol incorporation into total chloroform extracts and PI and phosphatidylinositol 4-phosphate was decreased in the sodium butyrate-treated cells. Moreover, the accumulation of the rapid histamine-stimulated phosphoinositide metabolites, i.e., inositol monophosphate, inositol diphosphate, and inositol triphosphate (IP_3) was decreased in these cells. To elucidate whether the decreased inositol phosphates were due to a decrease in the phosphoinositides hydrolysis, we measured the transient IP_3 production directly by a receptor-binding assay. Our results indicated that histamine-stimulated transient IP_3 formations were decreased. Taken together, these results indicated that multiple changes by multiple mechanisms of phospholipid metabolisms were found in sodium butyrate-treated C6 glioma cells. The decreased IP_3 formation and its subsequent action, i.e., Ca^{2+} mobilization, may play an early but pivotal role by which sodium butyrate induces C6 glioma cell differentiation.
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Sodium butyrate is known to stimulate differentiation of mouse erythroleukemic Friend cells (1), and regulate gene expression and cell cycle in HeLa cells (2). It has been shown to effect morphological and protein level changes in C6 glioma cells (3–5). By stimulating the activities of CoA-ligase in an oligodendroglial cell line (CB-II), our earlier studies indicated that sodium butyrate's ability to induce morphological transformation was correlated with an increase in the incorporation of [^{3}H]arachidonic acid into phosphatidylcholine (PC) and a decrease in the labeling of phosphatidylethanolamine (PE) (6,7). However, information is lacking concerning the effect of sodium butyrate on phosphoinositide metabolism and differentiation in C6 glioma cells. Recently, the decreases in phosphoinositide metabolism have been shown to correlate with cell differentiation (8–11). Our results pointed out that the incorporation of [^{3}H]arachidonic acid into various classes of phospholipids was altered in the sodium butyrate-treated C6 glioma cells. A greater proportion of [^{3}H]arachidonic acid has been found in the phosphatidylinositol (PI) in C6 glioma cells than in the PI in CB-II cells. Sodium butyrate caused an increase in the incorporation of arachidonic acid into PC and a decrease in PI and PE. Thus, changes in the phospholipid metabolism could play a role in C6 differentiation. In this study, we examine the phospholipid metabolism and the agonist-stimulate phosphoinositide hydrolysis in sodium butyrate-treated C6 cells.

MATERIALS AND METHODS

Materials. AG 1-X 8 resin (100–200 mesh, formate form) was purchased from BIO-RAD (Hercules, CA). Adenosine triphosphate (ATP), bovine serum albumin (BSA, essential fatty acid free), carbachol, histamine, L-glutamic acid-γ-monohydroxamate, hydroxylamine, imidazole-HCl, phenylmethanesulfonyl floride (PMSF), PC, PI, pyruvate kinase, sodium butyrate, sodium chloride and L-glutamate, were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Arachidonic acid (specific radioactivity, 100 Ci/mmol), and myo-[2-^{3}H]inositol (specific radioactivity, 20.5 Ci/mmol) were purchased from NEN/DuPont (Wilmington, DE). Ham's F10 medium was purchased from Gibco BRL (Gaithersburg, MD), and fetal bovine serum (FBS) was obtained from Hyclone Laboratories Inc. (Logan, UT) and PAA laboratory (Linz, Austria). Gentamicin was purchased from JRH Biosciences (Lenexa, KS). Organic solvents and high-performance thin-layer chromatograph plates (HPTLC, Kieselgel 60, 10 × 10 cm) were purchased from E. Merck (Darmstadt, Germany). Tissue culture plasticware was purchased from Corning (Corning, NY).

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Abbreviations: BSA, bovine serum albumin; CB-II, cerebellum derived clonal oligodendrocyte cell line; FBS, fetal bovine serum; HPTLC, high-performance thin-layer chromatography; IP, inositol monophosphate; IP_2, inositol 1,4-diphosphate; IP_3, inositol 1,4,5-triphosphate; LiCl, lithium chloride; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; NaB, sodium butyrate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEpl, plasmalogen phosphatidylethanolamine; PI, phosphatidylinositol; PI hydrolysis, phosphoinositides hydrolysis; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-diphosphate; TCA, trichloroacetic acid.

Cell culture. Later passages (>300) of C6 glioma cells originally obtained from the American Type Culture Collection (Rockville, MD) were stock cultured in T-75 flasks as monolayers in culture medium (Ham's F10 medium supplemented with 10% FBS and 50 µg/mL gentamicin) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. At confluence, the cells were rinsed with 0.02 M phosphate buffer (pH 7.4) containing 0.1% glucose (PBG) and then dissociated with 3 mL trypsin-EDTA (Gibco) for 3 min at 37°C. The cells were resuspended in culture medium, and aliquots of this suspension were then transferred to T-75 flasks or 60-mm dishes for the experiment.

Glutamine synthetase activity. Glutamine synthetase activity was determined according to the method of Palmiljan *et al*. (12). Cells were incubated in culture medium in the presence of 2.5 mM sodium butyrate for 12–48 h. The incubations were terminated by aspirating the media, and the cells were rinsed with PBG, scraped by a rubber policeman, and then transferred to a test tube. The cells were centrifuged at $200 \times g$ for 10 min, resuspended in imidazole-HCl buffer (0.05 M imidazole pH 7.2, 0.1% Triton X-100), and homogenized by a polytron for 10 s at 0°C. The homogenates were then centrifuged at $100{,}000 \times g$ at 4°C for 30 min, and the resultant supernatant was removed as the cytosolic fraction. The cytosolic protein concentrations were determined by Lowry's method, and the protein concentrations were adjusted with imidazole buffer to a final concentration of 1 µg/µL. Each of the cytosolic samples (100 µL) was then mixed with 900 µL of reaction buffer (0.2 M $MgCl_2$; 0.95 M glutamate; 2 M hydroxylamine; 0.5 M 2-mercaptoethanol; 0.2 M 2-phosphoenol pyruvate; 1 mg/mL pyruvate kinase; 10 mM ATP) and incubated at 37°C for 15 min. After incubation, the reaction was stopped by the addition of 1.5 mL ferric chloride buffer consisting of 0.37 M $FeCl_3$, 0.67 N HCl, and 0.2 M trichloroacetic acid (TCA). The optic density of glutamine was measured by spectrophotometry at wavelength 535 nm, and the result was expressed as percentage of the control.

The [^{3}H]arachidonic acid incorporation assay. The incorporation of [^{3}H]arachidonic acid into intact C6 cells was conducted by subculturing the cells in culture medium in 60-mm dishes for 1 d. The culture medium was then changed to fresh culture medium in the presence of 0–5 mM sodium butyrate for 22 h. The culture medium was removed, and [^{3}H]arachidonic acid (0.5 µCi per dish) in Ham's F10 medium supplemented with 2.5% FBS (the label medium) and 0–5 mM sodium butyrate was added. At the end of this 2-h labeling process, the label medium was removed, and the reaction was stopped by adding 1.3 mL of ice-cold methanol to the cultures.

The [^{3}H]arachidonic acid acylation. In the assay system, cells were cultured in T-75 flasks in culture medium for at least 3 d. Subconfluent cultures were removed by trypsin, and cell number and protein determined. After washing with PBG, cells were suspended (2×10^6/mL) in 50 mM Tris-HCl (pH 7.4) and were homogenized by a Potter-Elvehjem homogenizer (Tri-R Instrument Rockville Center, NY). Aliquots of 0.5 mL cell homogenate were incubated with 1 µCi [^{3}H]arachidonic acid, 0.1% fatty acid-free BSA, 0.1 mM CoA, 0.3 mM dithiothreitol, 2.5 mM ATP, 10 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.4 in the absence or the presence of sodium butyrate to a total of 1 mL per test tube as described earlier (7) and according to the method of Lin *et al*. (13). All incubations were carried out at 37°C for 5 min, and 4 µM LPI, 20 µM LPC, and 20 µM bovine brain LPE were used (7). The lipids, cofactors, and sodium butyrate together with cell homogenates in buffer were kept in an ice-cold water bath and vortexed vigorously for 20 s prior to incubation. After incubation, the reaction was stopped by adding 4 vol of chloroform/methanol (2:1, vol/vol).

Short-term [^{3}H]thymidine incorporation and myo-1, 2-[^{3}H]inositol incorporation into total chloroform extracts assays. The cells were cultured in T-75 flasks and pretreated with 2.5 mM sodium butyrate in culture medium for 24 h. The cells were harvested by trypsin, counted, and subcultured into 60-mm dishes at a density of 1×10^6/dish. The cells were then incubated with 0.5 µCi/mL [^{3}H]thymidine or 4 µCi/mL myo-1, 2-[^{3}H]inositol for 2 h in 5% CO_2 incubator at 37°C. The [^{3}H]thymidine incorporation reaction was terminated by aspiration, and the cells were scraped onto a test tube containing 500 µL 1% Triton X-100 plus 5 mL 6% TCA and incubated for 10 min at room temperature. Following incubation, the samples were transferred to a filtration unit with GF/C filter papers and rinsed five times with 6% TCA and once with 70% ethanol. The radioactivity of the filter papers was determined by scintillation spectrophotometry. The myo-1, 2-[^{3}H]inositol incorporation reactions were stopped by aspiration and the addition of 1.3 mL ice-cold methanol. The cells were scraped, lipids extracted, and the radioactivity of the chloroform extracts determined by scintillation spectrometry.

Prolonged myo-1, 2-[^{3}H]inositol incorporation into total chloroform extracts assays. The incorporation of [^{3}H]inositol into intact C6 cells was conducted by subculturing the cells in culture medium in 60-mm dishes for 1 d. The culture medium was then changed to medium supplemented with 2.5% FBS, in the presence of 4 µCi/mL [^{3}H]inositol, in the presence or absence of 2.5 mM sodium butyrate for 24 h. At the end of this 24-h labeling process, the reaction was stopped by aspiration and the addition of 1.3 mL ice-cold methanol. The cells were scraped, lipids extracted, and the radioactivity of the chloroform extracts determined by scintillation spectrometry.

Lipid extraction and separation. For lipid extraction, 4 vol of chloroform/methanol (2:1, vol/vol) were added to 1 vol of cell suspension. After mixing, the tubes were briefly centrifuged at 400 g to allow phase separation. The lower organic phases were removed and transferred to the test tubes. In order to obtain a complete recovery of the acidic phospholipids, the aqueous phase was further extracted by adding 2 vol of chloroform/methanol/12 N HCl (225:75:0.75, by vol). The acidic organic phase was removed and neutralized with 1 drop of 4 N NH_4OH before being recombined with the first organic extract. The combined organic extracts were evaporated to dryness, redissolved in chloroform, and applied to HPTLC

plates (Kieselgel 60, 10 × 10, E. Merck). Phospholipids, i.e., PI, PS, PC, PEpl and PE, were separated by a two-dimensional solvent system (7) according to the original method of Sun *et al.* (14). Briefly, plates were developed in the first dimension using chloroform/methanol/acetone/12 M NH_4OH (35:20:5:5, by vol). After development, the plate was removed from the tank, dried, and exposed to HCl fumes for 3 min before removing the HCl fumes by a stream of warm air for 10 min. The plates were then developed in the second dimension by chloroform/methanol/acetone/glacial acetic acid/0.2 M ammonia acetate (35:15:14:1:1.75, by vol). After development, the lipid fractions were visualized by exposure to iodine vapor, and the individual phospholipid spots were scraped into scintillation vials for counting.

For separation of phosphoinositides (PI, PIP, and PIP_2), the lipids were spotted on HPTLC Silica gel plates (E. Merck). These plates were impregnated with 1% potassium oxalate dissolved in methanol/H_2O (3:2, vol/vol) containing 2 mM EDTA. The plates were developed in a first solvent system containing chloroform/methanol/acetone/16 M NH_4OH (70:40:10:10, by vol). After development, the solvent was removed from the plates by blowing with an air gun for 5 min. The HPTLC plates were then developed in the same direction with a second solvent system containing chloroform/methanol/16 M NH_4OH/H_2O (36:28:2:6, by vol) according to Lin *et al.* (13). After the second solvent development, the plates were exposed to iodine vapors, the corresponding lipid bands were scraped, and radioactivity measured as described above.

Phosphoinositide hydrolysis and IP accumulation assay. Phosphoinositide hydrolysis was assessed by measuring the accumulation of IP in cells prelabeled with myo-1, 2-[^{3}H]inositol for 24 h. Cells (1×10^6 cells) were subcultured on T-25 flasks or 60-mm dishes and were incubated with 2 mL of labeled medium (F10 supplemented with 2% FBS and 4 μCi/mL of myo-1, 2-[^{3}H]inositol), in the presence of 2.5 mM sodium butyrate for 24 h in 5% CO_2 at 37°C. LiCl (10 mM) was added into the label medium 1 h before termination of the reaction. At termination, cells were then washed three times with assay buffer (111 mM NaCl; 26.2 mM $NaHCO_3$; 1 mM NaH_2PO_4; 2.5 mM KCl; 1.5 mM $MgSO_4$; 20 mM glucose; 10 mM LiCl; pH 7.4) and then incubated with histamine at a final concentration of 30 μM for 1–30 min at 37°C. The reaction was stopped by aspiration followed by the addition of ice-cold methanol. The accumulations of glycerol phosphoinositide (GPI), IP, inositol 1,4-diphosphate (IP_2), and inositol 1, 4, 5-triphosphate (IP_3) were assayed by using AG-1X 8 (formate form, 100–200 mesh; BIO-RAD) column and were eluted with 5 mM sodium borate/60 mM sodium formate, 0.2 N ammonium formate/0.1 N formic acid, 0.4 N ammonium formate/0.1 N formic acid, and 1 N ammonium formate/0.1 N formic acid to separate GPI, IP, IP_2, and IP_3, respectively, according to Berridge (15). In order to standardize the data, the total chloroform extracts of these samples were saved and the radioactivity measured.

Determination of inositol 1,4,5 P_3 (IP_3) mass by radioreceptor binding assay. The procedure for determination of IP_3 concentration in cells followed the method originally described by Bredt *et al.* (16) with modifications (17). In brief, cellular IP_3 was extracted by TCA from the supernatants and by washing five times with 2 vol of water-saturated diethyl ether. Residual ether was then removed by blowing with N_2 for 15 min, and the remaining cell extract was neutralized with 500 mM Tris buffer (pH 8.4) and stored at −80°C. The membranes containing receptors for the binding assay were isolated by homogenizing rat cerebella in 30 vol of Tris buffer (50 mM, pH 7.7) containing EDTA (1 mM) and 2-mercaptoethanol (1 mM) followed by centrifugation for 15 min at 4°C. The pellets were washed three times, resuspended in the same buffer (final protein concentration 4 mg/mL), and stored at −80°C. The concentrations of IP_3 were determined by incubating cell extracts and cerebellar membranes in Tris buffer (50 mM, pH 8.4) containing 1 nM [^{3}H]IP_3 (17 Ci/mmol). A standard curve was constructed using unlabeled IP_3 (1–100 nM) to replace cellular IP_3, and nonspecific binding was determined in the presence of 2 μM IP_3. The mixture was incubated at 4°C for 15 min, and the reactions were terminated by centrifugation at $15{,}000 \times g$ for 5 min. The pellets were solubilized with NaOH and neutralized with HCl, and radioactivity was determined by liquid scintillation spectrometry.

RESULTS

Effect of sodium butyrate on glutamine synthetase activities. To elucidate the effect of sodium butyrate on C6 glioma cells, the cytosolic glutamine synthetase activities were measured in the cells pretreated with 2.5 mM sodium butyrate for 0–48 h. As shown in Figure 1, increases in glutamine synthetase activities were detected in cells treated with sodium butyrate for 24–48 h, but not in cells treated with sodium butyrate for 12 h, suggesting that a longer time treatment is required for this induction.

Effect of sodium butyrate on [^{3}H]arachidonic acid incorporation into C6 glioma cells. The effect of sodium butyrate on phospholipid metabolism was studied by analysis of the arachidonic acid incorporation in these cells. When control C6 cells were labeled with [^{3}H]arachidonic acid for 2 h, 50% of the radioactivity was found in PC and 30% in PI. Less than 10% radioactivity was found in PE and less than 5% in PS, and PEpl in these cells. As shown in Table 1, sodium butyrate stimulated increases in the labeling of PC and decreases in the labeling of PI and PE. In cells treated with 2.5 mM sodium butyrate for 24 h, the labeling of PC was increased 28% and the labeling of PI was decreased 30%, suggesting that sodium butyrate may alter the acylation of arachidonic acid among these phospholipids differently.

Effect of sodium butyrate on acylation of [^{3}H]arachidonic acid. To further elucidate the changes in PC, PE, and PI labeling, we analyzed the incorproation of [^{3}H]arachidonic acid by an acylation assay. As shown in Table 2, the amount of [^{3}H]arachidonic acid in PI in the absence of exogenous substrates was three times greater than PC and PE by this assay. In the presence of LPI, LPC, and LPE, the labeling of PI, PC,

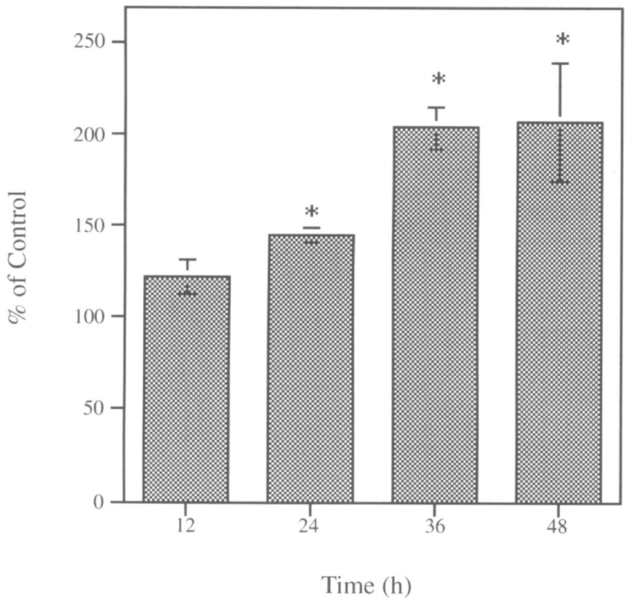

FIG. 1. The effect of sodium butyrate on glutamine synthetase activity of C6 cells. Cells were cultured in the absence (control) or the presence of 2.5 mM sodium butyrate for 12–48 h as indicated. The cytosolic glutamine synthetase activities were assayed as described in the Materials and Methods section. Data represent mean ± SD from three determinations and the symbol * denotes a significantly different mean from the controls as determined by nonpaired Student *t*-test with $P \leq 0.05$.

and PE was increased about 2-fold, 6-fold, and 3-fold, respectively. The addition of 2.5 mM sodium butyrate to the incubation system further increased the labeling of PI (1.7-fold), PC (1.7-fold), and PE (1.6-fold). These results indicated that C6 cells contain a very active alternative biosynthesis system to transfer arachidonic acid into LPI, LPC, and LPE for the synthesis of PI, PC, and PE.

Effect of sodium butyrate on the incorporation of [^{3}H]glycerol into phospholipids. To further elucidate the effect of sodium butyrate on the biosynthesis of phospholipids, cells were labeled with [^{3}H]glycerol in the absence or the presence of sodium butyrate. As shown in Table 3, a major proportion of radioactivity was found in PC, and lesser amounts were found in PI and PE. The labeling of PC and PE was only slightly altered in the sodium butyrate-treated cells, indicating that sodium butyrate may not affect the *de novo* phospholipid biosynthesis pathway in these cells.

Effect of sodium butyrate on [^{3}H]thymidine incorporation, protein concentration, and [^{3}H]inositol incorporation into total chloroform extracts by a short-term assay. To further elucidate the timing of the onset of sodium butyrate-induced C6 cell differentiation and to correlate it with changes in phosphoinositides metabolism, we monitored [^{3}H]thymidine incorporation, protein concentrations, and [^{3}H]inositol incorporation into total chloroform extracts on C6 cells pretreated with 2.5 mM sodium butyrate for 0, 2, 6, 12, and 24 h. As shown in Table 4, the onset of decreases in [^{3}H]thymidine incorporation and [^{3}H]inositol incorporation were observed in cells pretreated with sodium butyrate for 6 h, indicating that an initial effect must have occurred within 6 h. Equilibrium may be reached between 12 and 24 h in these cells.

Effect of sodium butyrate on [^{3}H]inositol incorporation into PI, PIP, and PIP_2 of C6 glioma cells. The effect of sodium butyrate on prolonged [^{3}H]inositol incorporation into PI, PIP, and PIP_2 was further elucidated by TLC analysis of the chloroform extract. As shown in Figure 2, the [^{3}H]inositol incorporation into PI and PIP was significantly lower in the sodium butyrate-treated C6 cells.

Effect of sodium butyrate on IP, IP_2, and IP_3 accumulation. To correlate the changes in phosphoinositide biosynthesis associated with reduction in the phospholipid-derived second-messenger productions, we measured the accumulation of inositol IP, IP_2, and IP_3 in sodium butyrate-treated C6 cells. A time-course study of the accumulation of IP, IP_2, and IP_3 (Fig. 3) showed decreasing levels of IP, IP_2, and IP_3.

Effect of sodium butyrate on agonist-stimulated inositol IP_3 production. The effect of sodium butyrate on the transient IP_3 production was further investigated by a receptor binding assay. A time-course study (5–30 s) was carried out in which cells were previously treated with 2.5 mM sodium butyrate for

TABLE 1
Concentration Effect (% of total) of Sodium Butyrate on [^{3}H]Arachidonic Acid Incorporation into Phospholipids of C6 Glioma Cells[a,b,c]

NaB (mM)	0	1	2.5	5
Phospholipid				
PI	31.1 ± 0.52	25.9 ± 0.95[d]	21.6 ± 0.26[d]	19.0 ± 0.64[d]
PS	4.19 ± 0.12	5.46 ± 0.52[d]	4.22 ± 0.22	3.94 ± 0.15
PC	50.8 ± 0.90	57.9 ± 1.52[d]	65.2 ± 0.56[d]	68.6 ± 1.60[d]
PEpl	3.98 ± 0.23	4.14 ± 0.57	3.86 ± 0.24	3.78 ± 0.68
PE	9.93 ± 0.13	6.61 ± 1.06[d]	5.19 ± 0.29[d]	4.18 ± 0.97[d]
Total dpm	66355 ± 4257	68145 ± 3004	57391 ± 3118[d]	59195 ± 1037[d]

[a]The C6 cells were cultured in F-10 medium supplemented with 10% fetal bovine serum in 60-mm dishes, treated with the indicated concentration of sodium butyrate (NaB) for 24 h and labeled with 0.5 μCi [^{3}H]-arachidonic acid for 2 h as described in the Materials and Methods section.
[b]Radioactivity of the lipids in disintegrations per minute (dpm) was expressed as percentage of total (means ± SD) from three determinations.
[c]Lipids were separated into phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), plasmalogen phosphatidylethanolamine (PEpl), and phosphatidylethanolamine (PE) by a two-dimensional method and radioactivity measurement as described in the Materials and Methods section.
[d]Denotes values significantly different from controls ($P \leq 0.05$), based on nonpaired Student's *t*-test.

TABLE 2
Effect of Sodium Butyrate (dpm) on [^{3}H]Arachidonic Acid Incorporation into Phospholipids of C6 Cells Homogenates [a,b,c]

Lyso-PL	–		+		+	
NaB	–		–		+	
Phospholipid						
PI	15051 ± 1196	(100)	32041 ± 2557[d]	(213)	55245 ± 1841[d]	(367)
PC	5012 ± 637	(100)	28618 ± 2800[d]	(571)	49198 ± 5159[d]	(982)
PE	5630 ± 637	(100)	18252 ± 1937[d]	(324)	29084 ± 897[d]	(517)

[a]The C6 cells were cultured in F-10 medium supplemented with 10% fetal bovine serum for 2 d in T-75 flasks. The cells were removed, washed, and homogenized in Tris-buffer (50 mM, pH 7.4), and aliquots of cell homogenates were taken for *in vitro* [^{3}H]arachidonic acid incorporation analysis in the presence of lysophospholipids (Lyso-PL), 2.5 mM sodium butyrate (NaB), and further incubated at 37°C for 5 min.
[b]Radioactivity of the lipids in disintegrations per minute (dpm) was expressed as percentage of total (means ± SD) from three determinations. The numbers in parentheses represent the percent of the labeled phospholipids relative to the -Lyso-PL and -NaB controls.
[c]Lipids were separated into phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) by a two-dimensional method and radioactivity measurement as described in the Materials and Methods section.
[d]Denotes values significantly different from no treatment controls ($P \leq 0.05$) in each row based on nonpaired Student's *t*-test.

24 h. The results demonstrated that histamine stimulated IP_3 production at 5 s and reached a maximum within 10 s. As shown in Figure 4, IP_3 production was decreased almost to the basal level in the sodium butyrate-treated cells. To further confirm that the decreased IP_3 production is from PI turnover, we subsequently measured the temporal response of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in these cells by the Fura-2 method. Results indicated that the $[Ca^{2+}]_i$ concentrations were decreased in the sodium butyrate-treated C6 cells 5 s after the addition of the histamine (data not shown), suggesting that the decreased IP_3 resulted from a decreased PI hydrolysis.

DISCUSSION

The glutamine synthetase (GS) (E.C. 6.3.1.2) has been used as a marker for C6 cells (18) and astrocytes in rat brain (19, 20). An early report has shown that sodium butyrate alone increased GS protein synthesis through stimulation of post-transcriptional regulation in C6 cells (21). In this study, treating C6 cells with sodium butyrate for 24 h induced an increase in GS activities (Fig. 1), suggesting that the treatment induced cell differentiation. An initial increase was obtained after 24 h and maximal increase obtained after 36 h. This result correlated with a recent report indicated that staurosporine induced differentiation of C6 cells and an initial increase in GS activity obtained after 24 h and maximal effect obtained between 24–48 h (22). A similar initial effect but a delayed (72 h) maximal effect was also found in the interleukin-4 induced C6 differentiation (23).

To determine whether the effect of sodium butyrate on phospholipid metabolism is associated with C6 differentiation, we analyzed the incorporation of arachidonic acid, glycerol, and inositol into these cells. Our results demonstrated that, similar to our earlier findings in an oligodendrocyte cell line (CB-II) (7), sodium butyrate increased the incorporation of arachidonate into PC and decreased its incorporation into PE. On the other hand, butyrate stimulated arachidonate incorporation into LPC, LPI, and LPE. Thus butyrate may also affect the turnover of arachidonate in these cells. The PE decrease was probably due to an inhibition of the transfer of arachidonic acid from PC to PE by a transacylation mechanism. An earlier time-course study in CB-II oligodendrocytes has shown that arachidonic acid was rapidly incorporated into PC (6). Compared with PC,

TABLE 3
Concentration Effect (% of total) of Sodium Butyrate on [^{3}H]Glycerol Incorporation into Phospholipids of C6 Glioma Cells[a,b,c]

NaB (mM)	0	1	2.5	5
Phospholipid				
PI	9.61 ± 0.50	10.3 ± 0.83	9.37 ± 0.66	9.35 ± 0.93
PS	6.62 ± 0.61	6.56 ± 0.74	5.29 ± 0.30[d]	4.84 ± 0.65[d]
PC	68.6 ± 0.61	67.7 ± 1.31	70.9 ± 1.37	72.0 ± 1.39[d]
PEpl	2.03 ± 0.12	2.34 ± 0.51	2.97 ± 0.41[d]	2.99 ± 0.44[d]
PE	13.1 ± 0.46	13.1 ± 0.15	11.5 ± 0.40[d]	11.2 ± 0.32[d]
Total dpm	171739 ± 5624222	175821 ± 12590	177869 ± 9129	181062 ± 12851

[a]The C6 cells were cultured in F-10 medium supplemented with 10% fetal bovine serum in 60 mm dishes, the indicated concentration of sodium butyrate (NaB) and 1 μCi [^{3}H]-glycerol/mL for 24 h in the label medium in the presence of the indicated concentration of NaB as described in the Materials and Methods section.
[b]Radioactivity of the lipids in disintegrations per minute (dpm) was expressed as percentage of total (means ± SD) from three determinations.
[c]Lipids were separated into phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), plasmalogen phosphatidylethanolamine (PEpl), and phosphatidylethanolamine (PE) by a two-dimensional method and radioactivity measurement as described in the Materials and Methods section.
[d]Denotes values significantly different from controls ($P \leq 0.05$), based on nonpaired Student's *t*-test.

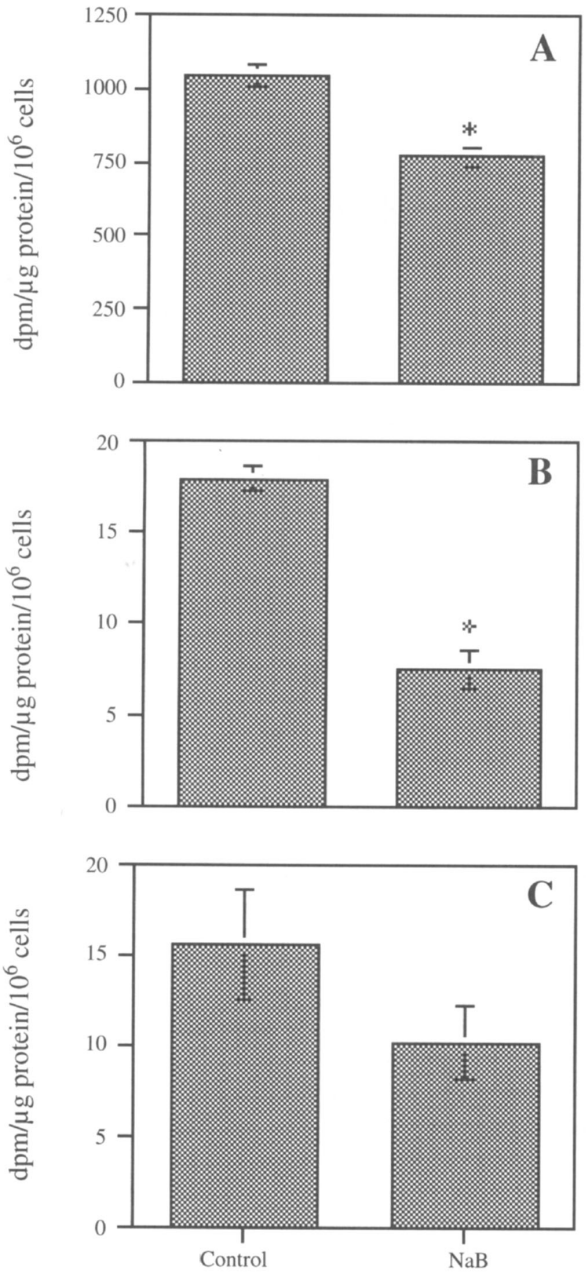

FIG. 2. Effect of sodium butyrate on [^{3}H]inositol incorporation into phosphoinositides of C6 cells. Cells were cultured in the absence (control) or the presence of 2.5 mM sodium butyrate (NaB) for 24 h. Lipids were extracted and separated and the radioactivity of (A) PI, (B) PIP, and (C) PIP_2 determined by scintillation spectrometry. The values, standardized as dpm/µg protein/10^6 cells, are mean ± SD from three determinations and the symbol * indicates a significant difference from the controls.

TABLE 4
Effect of Sodium Butyrate on [^{3}H]Thymidine Incorporation, Protein Levels, and Incorporation of [^{3}H]Inositol into Total Chloroform Extracts of C6 Cells[a]

	Thymidine[b] incorporation (% of control)	Protein[c] levels (µg)	Inositol[d] incorporation (dpm/100 µg protein)
Control	100	153 ± 5	4032 ± 337
NaB 2 h	n.d.[f]	158 ± 31	4093 ± 241
NaB 6 h	74 ± 10[e]	223 ± 4[e]	2316 ± 178[e]
NaB 12 h	51 ± 4[e]	226 ± 6[e]	1898 ± 257[e]
NaB 24 h	58 ± 3[e]	241 ± 10[e]	1669 ± 421[e]

[a]The cells were cultured in F-10 media supplemented with 10% fetal bovine serum (FBS) in the absence (control) or the presence of 2.5 mM sodium butyrate (NaB) for the indicated lengths of time. Cells were then harvested, counted and 10^6 cells were taken for the subsequent experiments.
[b]The thymidine incorporation was determined as described in Materials and Methods by resuspending 10^6 cells with 0.5 µCi/mL [^{3}H]thymidine and incubating for 2 h. Data represent mean ± SD from six determinations.
[c]Protein levels were analyzed from 10^6 cells by Lowry's method (Ref. 30). Data represent mean ± SD from three determinations.
[d]Cells were resuspended in F10 media supplemented with 2.5% FBS and 4 µCi/mL [^{3}H]inositol with a density of 10^6 cells per dish and cultured in 6-cm dishes for 2 h. The reactions were stopped by aspiration, and the addition of 1.3 mL of ice-cold methanol. The lipids were extracted and the radioactivity of the total chloroform extracts determined by scintillation spectrometry. Data were standardized as dpm/100 µg protein and represent mean ± SD from six determinations.
[e]Denotes a significantly different mean as compared with the controls in each column by nonpaired Student's *t*-test with $P \leq 0.05$.
[f]Not detected.

the incorporation of arachidonic acid into PE was slow and had a late onset. It is possible that arachidonate was transfered from PC to PE in C6 cells, and sodium butyrate could inhibit this process. To investigate this possibility, we incubated arachidonic acid-prelabeled C6 cells in culture medium in the presence of sodium butyrate, and found that arachidonic acid was transferred from PC to PE and PI. Sodium butyrate decreased PE but not PI labeling in these cells as compared with the control cells (data not shown). This PE-transacylation pathway remains to be more clearly characterized.

Compared with CB-II cells (6, 7), [^{3}H]arachidonic acid was more actively incorporated into PI in C6 cells. Radioactivity of PI decreased in the sodium butyrate-treated C6 cells but not CB-II cells. In the absence of exogenous lysophospholipids, the amount of radioactivity found in PI was three times that in PC and PE (Table 2), suggesting that there was abundant endogenous LPI in these cells. Based on this finding, the differences in the distribution of radioactivity of [^{3}H]arachidonic acid may be mostly due to the availability of the endogenous lysophospholipids. The results also imply that C6 membrane contains enzymes (i.e., CoA–ligase and acyltransferase) to synthesize PI, PC, and PE. When substantial amounts of exogenous LPI, LPC, and LPE were added into the incubation system, a respective 2-fold, 6-fold, and 3-fold increase in the labeling of PI, PC, and PE was observed. This result confirms our earlier observation that acylation of these phospholipids was very active in the glial cells. It may also provide support for the widely held belief that different lysophospholipid acyltransferases are involved with acylation of PC, PE, and PI (24). Since there is 4–6 times as much PC as PI, the turnover of PI may be higher than PC in C6 cells. As shown in Table 2, sodium butyrate stimulated a similar fold of increase (1.7-fold) in the transfer of arachidonate into LPI, LPC, and LPE. Taken

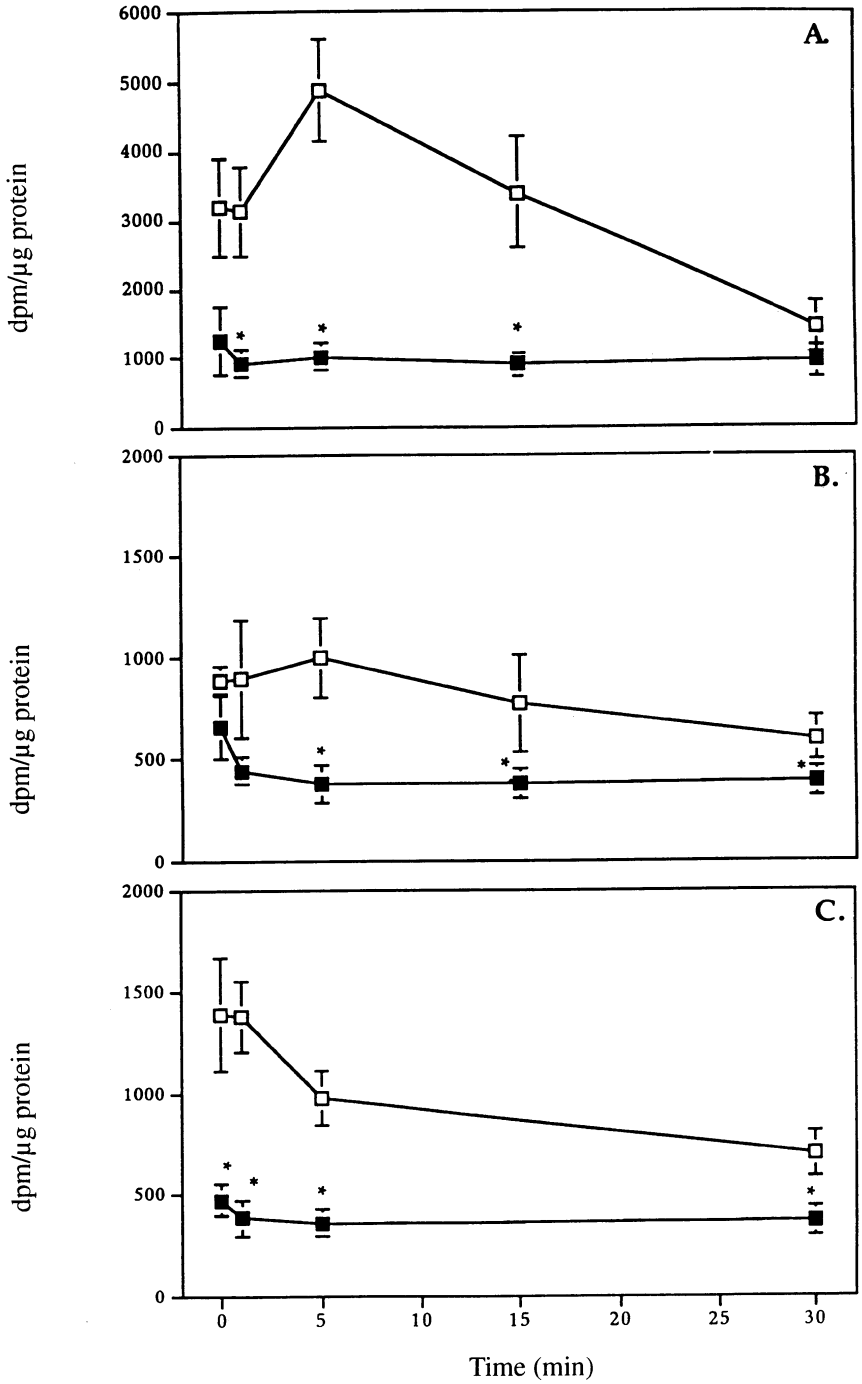

FIG. 3. Effect of sodium butyrate on histamine-stimulated accumulation of (A) IP, (B) IP_2, and (C) IP_3. C6 cells were seeded in 60-mm dishes and preincubated with 4 µCi/mL [^{3}H]inositol in the absence (□) or the presence (■) of 2.5 mM sodium butyrate for 24 h. The medium was removed and the cells were stimulated with 30 µM of histamine for the indicated time period (1–30 min) before being eluted by Dowex column. Radioactivity was determined by scintillation spectrometry as described in the Materials and Methods section. Data (dpm/100 µg protein) represent mean ± SD from three determinations and the symbol * denotes a significantly different mean from the respective control (i.e., at the same time point) by nonpaired Student's *t*-test with $P \leq 0.05$.

together, when lysophospholipids were available, sodium butyrate stimulated a similar mode of increase in the transfer of arachidonic acid into LPI, LPC, and LPE through the stimulation of a common mechanism, the CoA–ligase (7).

We have demonstrated that inositol incorporation into total chloroform extracts and TLC-separated PI and PIP were significantly lower in the sodium butyrate-treated C6 cells, suggesting that the biosynthesis of phosphoinositides was

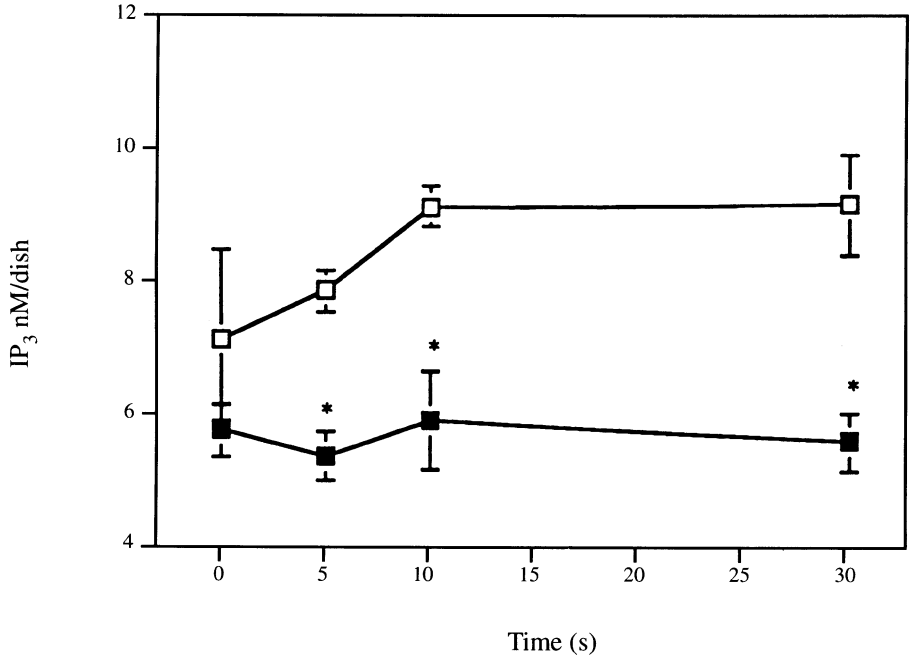

FIG. 4. Effect of sodium butyrate on histamine-stimulated inositol 1,4,5 triphosphate (IP_3) production. The C6 cells were seeded in 35-mm dishes and preincubated in the absence (□) or the presence (■) of 2.5 mM sodium butyrate for 24 h. The media were removed and the cells rinsed and incubated with 30 μM of histamine for the indicated time periods (0–30 s). The IP_3 was measured by radioreceptor binding assay as described in the Materials and Methods section. Data, nM/dish, represent mean ± SD from three determinations and the symbol * denotes a significantly different mean from the control at the same time point by nonpaired Student's *t*-test with $P \leq 0.05$.

decreased in these cells. Short-term (30–120 min) treatment did not affect thymidine incorporation in these cells (data not shown). A sodium butyrate time-course study, which was conducted by incubating the same number of cells (1×10^6) with [^{3}H]thymidine for 2 h, indicated that the decrease in thymidine incorporation is time- (Table 4) and concentration-dependent (data not shown). The time-course study further indicated that the onset of decrease in thymidine incorporation started as early as 6 h after the addition of sodium butyrate and was correlated with decreased inositol incorporation. This finding makes it likely that the real effect of butyrate on C6 differentiation and decrease in inositol incorporation would have occurred even earlier.

The decreased IP_3 in the sodium butyrate-treated cells indicated that PI hydrolysis and the generation of phospholipid-derived second messengers were affected in these cells. In addition to the generation of IP_3 reported in this study, we have confirmed that histamine also stimulated a transient Ca^{2+} response *via* H_1 receptors 5 s after the addition of agonists (data not shown). This would agree with the earlier findings by others that histamine H_1-receptor mediated PI hydrolysis in primary astrocyte culture (25) and in 131N1 astrocytoma cells (26). Furthermore, it is also in agreement with another finding in UC-11MG astrocytes that histamine stimulates a significant Ca^{2+} response *via* H_1 receptors (27,28). In this study, two different assay methods both showed that histamine-stimulated rapid accumulation of IP_3 was decreased in sodium butyrate-treated cells. Moreover, the transient histamine-stimulated Ca^{2+} mobilization was also decreased although the H_1 receptors were actually increased in the sodium butyrate-treated cells (data not shown). Thus, by eliminating the possibility that decreased phosphoinositide hydrolysis was due to changes in receptor, these results confirm that decreased phosphoinositide hydrolysis reflects changes in phosphoinositide metabolism. Since sodium butyrate did not decrease phosphoinositides labeling when cells were incubated with [^{3}H]glycerol for 24 h, this result suggests that butyrate does not decrease the mass of inositol-containing lipids. However, that sodium butyrate decreased the inositol incorporation of PI and PIP suggests that sodium butyrate must affect the *de novo* synthesis of inositol-containing lipids. Furthermore, there is a 59% decrease in inositol incorporation into total chloroform extracts by short-term incorporation. As short-term inositol and arachidonate incorporation, baseline mass of the inositol phosphate second messengers, the inositol phosphate response to histamine, and the subsequent Ca^{2+} mobilization are all reduced, it seems likely that butyrate decreases the turnover of agonist-sensitive pool of phosphoinositide. Our results are similar to those of Ponzoni and Lanciotti (9) and Lanciotti and Longone (29), who have reported a retinoic acid-induced Lan-1 neuroblastoma cell differentiation involving a rapid decrease of inositol

phospholipid turnover.

In conclusion, we have shown that sodium butyrate-induced glial cell differentiation is accompanied by enhanced arachidonic acid incorporation into PC and decreases in phosphoinositide turnover. Sodium butyrate may also inhibit the transfer of arachidonic acid from PC to PE. The decreases in phosphoinositide turnover lead to decreases in the generation of the phospholipid-derived second messengers, IP_3 and its subsequent action, the Ca^{2+} mobilization. Taken together, these results provide evidence that sodium butyrate-stimulated C6 cell differentiation is correlated with multiple changes in phospholipid metabolism, the production of their metabolites, and the physiological action of the metabolites by which sodium butyrate signals may be transduced. Thus, changes in phospholipid may play an early but pivotal role in the sodium butyrate-induced glioma cell differentiation.

ACKNOWLEDGMENTS

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Polyunsaturated Fatty Acids Increase the Sensitivity of 36B10 Rat Astrocytoma Cells to Radiation-Induced Cell Kill

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ABSTRACT: Polyunsaturated fatty acids (PUFA) such as γ-linolenic acid (GLA, 18:3n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) have been shown to be cytotoxic to tumor cells. The objective of this work was to study the effects of PUFA on the radiation response of a 36B10 rat astrocytoma cell line. Supplementation of the astrocytoma cells with 15–45 μM GLA, EPA, or DHA produced marked changes in the fatty acid profiles of their phospholipids and neutral lipids. The methylene bridge index of these lipids increased significantly. These PUFA also exerted cytotoxic effects, as determined using the clonogenic cell survival assay. While GLA and DHA produced a moderate cell-killing effect, EPA was extremely cytotoxic, especially at a concentration of 45 μM. The monounsaturated oleic acid (OA, 18:1n-9) did not affect cell survival. Further, all three PUFA, and particularly GLA, increased the radiation-induced cell kill; OA did not enhance the effect of radiation. α-Tocopherol acetate blocked the enhanced radiation sensitivity of GLA- and DHA-supplemented cells. In conclusion, GLA, EPA, and DHA supplementation prior to, during, and after irradiation can enhance the radiation-induced cytotoxicity of rat astrocytoma cells. GLA and DHA supplementation post-irradiation also enhanced the radiation response of the 36B10 cells. Because GLA maximally increases the radioresponsiveness of a rat astrocytoma, this PUFA might prove useful in increasing the therapeutic efficacy of radiation in the treatment of certain gliomas.

Lipids 32, 283–292 (1997).

In the treatment of brain tumors with radiation, damage to the surrounding normal tissue is a major dose-limiting factor since injury to the brain could lead to severe functional disabilities in patients. Malignant gliomas are extremely radioresistant and hence particularly difficult to treat (1–3). Therefore, to increase the therapeutic efficacy of radiation in the treatment of these tumors, it is extremely important to selectively kill tumor cells.

In recent years, there has been a growing interest in the potential of polyunsaturated fatty acids (PUFA) to selectively enhance the response of tumors to various forms of anticancer therapy. Fatty acid supplementation produces changes in the lipid composition of cells which can alter their physical and functional properties (4). Further, several studies have shown that supplementation with PUFA can influence tumor incidence, growth, and metastasis (5–8).

The cytotoxic action of PUFA is thought to be mediated predominantly *via* the generation of free radicals and lipid peroxidation (9–11). Incorporation of PUFA into tumor cells leads to increased potential for oxidative stress (12) and increases the susceptibility of the cells to therapies that generate reactive oxygen species. For example, increased incorporation of PUFA leads to enhanced sensitivity of tumor cells to hyperthermia (4) and some anticancer drugs such as adriamycin and methotrexate (13,14). Since radiation also generates oxidative stress in cells, supplementation of membrane lipids with PUFA could augment radiation-induced cell kill. However, the effect of PUFA on the radiation response of tumor cells has not been well documented (15). The purpose of this study was to determine whether PUFA can alter the response of a malignant rat astrocytoma cell line to radiation.

MATERIALS AND METHODS

Cell and culture conditions. The cell line used was the ethylnitrosourea-induced 36B10 malignant rat astrocytoma (16). The 36B10 cells were cultured at 37°C under a humidified atmosphere of 95% air: 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and L-glutamine (2 mM), penicillin (50 IU/mL), and streptomycin (50 μg/mL). All cell culture reagents were purchased from Gibco (Grand Island, NY). Stock cultures were maintained as monolayer cultures in tissue culture flasks by routine passage twice a week by trypsinization.

Lipid extraction and assays. The fatty acid composition of 36B10 cells was determined by gas–liquid chromatography (17). Incorporation of fatty acids into the cells was determined by adding 15–45 μM of docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), or γ-linolenic acid (GLA, 18:3n-6) to the growth medium. The monounsaturated fatty acid, oleic acid (OA, 18:1n-9), was also used as a fatty acid control. Fatty acids were purchased

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Abbreviations: AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DTA, docosatetraenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid, OA, oleic acid; PUFA, polyunsaturated fatty acids.

from Cayman Chemical Company (Ann Arbor, MI). The fatty acid solutions were prepared in ethanol such that the concentration of alcohol in the medium was <0.2%. After incubation for 24 h, lipids were extracted using chloroform/methanol (2:1, vol/vol) following the standard protocol of Folch *et al.* (18). A measured quantity of heptadecanoic acid (17:0) was also added as an internal standard to quantify the amounts of fatty acids present in the samples.

Total cholesterol in the cell lipid extracts was determined using an enzymatic kit (Sigma Diagnostics, St. Louis, MO). The phospholipid content of samples was determined colorimetrically using the malachite green assay for inorganic phosphorus (19).

Neutral and phospholipid fractions were separated using silicic acid columns (20). Columns were prepared by washing with *n*-heptane. Lipid extracts suspended in chloroform/methanol (1:1, vol/vol) were loaded onto the column, and the column was washed with 1 mL heptane. Neutral lipids were eluted with 6 mL chloroform/methanol (100:2, vol/vol) and phospholipids with 8 mL methanol/distilled water (100:2, vol/vol).

For analysis of fatty acid composition, the lipid extracts were transesterified with 12% BF_3 in methanol at 95°C for 45 min (21). The resulting fatty acid methyl esters were extracted into *n*-heptane and separated using a Hewlett-Packard 5890 system (Palo Alto, CA) containing a 1.9 m × 2 mm glass column packed with 10% SP2330 on 1000/120 mesh Chromosorb WAW (Supelco, Bellefonte, PA). N_2 was used as the carrier gas at a flow rate of 25 mL/min. The column injector was maintained at 250°C while the oven temperature was programmed to remain at 175°C for 7 min and then increased to 240°C at 2°/min. Fatty acid methyl esters were detected by flame ionization at 250°C and quantified using an HP 3990A integrator. Chromatogram peaks were identified by comparison with the retention times of standards purchased from Nu-Chek-Prep (Elysian, MN).

For determination of methylene bridge index, the number of *bis*-allylic methylene bridge positions contained in each fatty acid methyl ester was multiplied by its respective nanogram quantity and summed for all fatty acids detected. The mean number of *bis*-allylic methylene bridge positions per fatty acyl chain was then expressed as the methylene bridge index.

Cell survival analyses. The radiation response of the 36B10 cells was determined using the clonogenic cell survival assay. A single cell suspension was obtained by trypsinization of 90% confluent cell cultures. Viable cells were counted using 0.4% erythrosin B with a hemocytometer (viability >95%) and seeded at varying densities (depending on radiation dose) onto 60 mm plates. After 2–3 h, the cells were exposed to 0–10 Gy γ-rays (^{137}Cs γ-source, 1.5 Gy/min). The cells were allowed to grow for one week, the medium being changed once every 2 d. At the end of this incubation, the cells were stained with 0.1% crystal violet for 60 min, and the colonies (>50 cells/colony) were counted using a dissecting microscope.

To determine the effect of fatty acids, the cells were incubated in medium supplemented with 15–45 μM GLA, EPA, DHA, or OA for 24 h prior to seeding and were continued on the fatty acid-supplemented media throughout the duration of the experiment. In order to investigate whether lipid peroxidation played a role in the effects of PUFA on the astrocytoma cells, 50 or 100 μM of α-tocopherol acetate was added to the media in addition to the fatty acids, and the clonogenic survival was determined. The cells were maintained in the antioxidant-rich media throughout the duration of the experiment. The effect of fatty acid supplementation post-irradiation was studied by incubating the cells in unsupplemented media prior to and during irradiation. Within 1 h of irradiation, the medium was replaced with fatty acid-supplemented media. Cells were then continued on supplemented media until the end of the experiment. To determine whether incubation of cells with fatty acids only prior to irradiation was sufficient to exhibit cytotoxic effects, and to study the influence of time between supplementation and irradiation, cells were incubated in fatty acid-supplemented media for 24 h. The cells were then returned to unsupplemented media. At intervals of 0, 24 and 48 h, the cells were seeded onto 60-mm plates and irradiated with 5 Gy γ-rays; control cells were treated similarly but not irradiated. Cells were then allowed to grow in unsupplemented media for one week and stained with 0.1% crystal violet for 60 min.

Statistical analysis. Statistical analysis of the radiation response data was performed using simple linear regression. Simple linear regression models of the form "log (surviving fraction) = β dose" were fitted. Student's *t*-tests were used to compare each slope to the control slope; P values ≤0.05 were considered to be statistically significant. D_0 values, defined as the dose (Gy) required to reduce the fraction of surviving cells to 37% of its previous value, were estimated by the negative reciprocal of the slope. Standard errors for D_0 values were computed from the standard errors of the estimated slopes using Taylor series variance approximations (the delta method). Statistical significance of data on fatty acid incorporation was analyzed using the Student's *t*-test. Data with P values ≤0.05 were considered to be statistically significant.

RESULTS

Incorporation of fatty acids. Table 1 shows the effects of 15–45 μM GLA, EPA, DHA, and OA supplementation on the total PUFA content of phospholipids and neutral lipids in rat astrocytoma cells. When the cells were supplemented with 15–45 μM OA, no changes in PUFA content of phospholipids or neutral lipids were observed. However, incubation of the cells with either GLA, EPA, or DHA led to dose-dependent increases in the total PUFA contents of the phospholipids and neutral lipids. As expected, the extent of increase was much more pronounced in the neutral lipids as compared with phospholipids, although the absolute amount was smaller. For example, supplementation of the astrocytoma cells with 45 μM GLA resulted in a 2-fold increase in the PUFA content of

TABLE 1
Effect of Fatty Acid Supplementation on the Total PUFA Content of Phospholipids and Neutral Lipids in 36B10 Astrocytoma Cells[a]

	Total PUFA content (nanograms/10^5 cells)	
Fatty acid	Phospholipids	Neutral lipids
GLA Controls	223.92 ± 10.28	13.32 ± 2.92
15 μM	367.79 ± 51.31*	66.80 ± 7.18**
30 μM	418 59 ± 43.51**	120.91 ± 15.01**
45 μM	470.90 ± 34.26**	190.50 ± 26.20**
EPA Controls	288.14 ± 24.68	13.68 ± 2.62
15 μM	346.23 ± 20.48	29.20 ± 1.13*
30 μM	417.09 ± 19.42**	24.94 ± 5.05*
45 μM	459.61 ± 26.38**	51.28 ± 10.91**
DHA Controls	286.97 ± 30.34	16.90 ± 4.29
15 μM	401.10 ± 20.85*	80.72 ± 8.52**
30 μM	472.63 ± 41.79*	256.19 ± 34.34**
45 μM	562.42 ± 60.26**	259.08 ± 22.66**
OA Controls	244.92 ± 15.26	trace
15 μM	226.26 ± 6.96	trace
30 μM	231.52 ± 14.25	trace
45 μM	248.56 ± 27.48	trace

[a]Values represent mean ± SE from four separate cultures. Cells were grown in 15–45 μM of the fatty acids for 24 h prior to lipid extraction. Statistical analysis was performed using Student's *t*-test; PUFA, polyunsaturated fatty acids. *$P < 0.05$ and **$P < 0.01$ significantly different from controls (no fatty acid supplement) Trace: Fatty acid was not detected in all samples; the amount was <5 nanograms when present.

phospholipids; the PUFA content in the neutral lipids increased about 14-fold. Similar increases in total PUFA content were observed for cells supplemented with EPA and DHA. Although the increases in total PUFA content were similar for cells supplemented with GLA, EPA or DHA, the specific nature of the changes depended on the fatty acid with which the cells were incubated.

Figure 1 illustrates the effect of incubation of cells with increasing concentrations of GLA. Supplementation with 15–45 μM GLA not only resulted in a dose-dependent increase in the content of this fatty acid but also elevated the levels of dihomo-γ-linolenic acid (DGLA, 20:3n-6) and to a lesser extent arachidonic acid (AA, 20:4n-6) and docosatetraenoic acid (adrenic acid; DTA, 22:4n-6) (Fig. 1). Thus, the total DGLA/AA ratio also increased in a dose-dependent manner from 0 in the control group to 0.32 in the cells incubated with 45 μM GLA. While the quantities of GLA, DGLA, AA, and DTA increased in the cell phospholipids, the amounts of other unsaturated fatty acids such as palmitoleic acid (16:1n-7), OA, and linoleic acid (18:2n-6) decreased when the cells were exposed to increasing GLA concentrations (data not shown). In the neutral lipids, however, only a decrease in palmitoleic acid was observed. Docosapentaenoic acid (22:5n-6) was not detected in any of the samples analyzed.

Figure 2 shows the fatty acid profiles of cells following 15–45 μM EPA supplementation. Cells incubated with EPA showed dose-dependent increases in quantities of EPA and DPA (22:5n-3) in their phospholipids and neutral lipids as

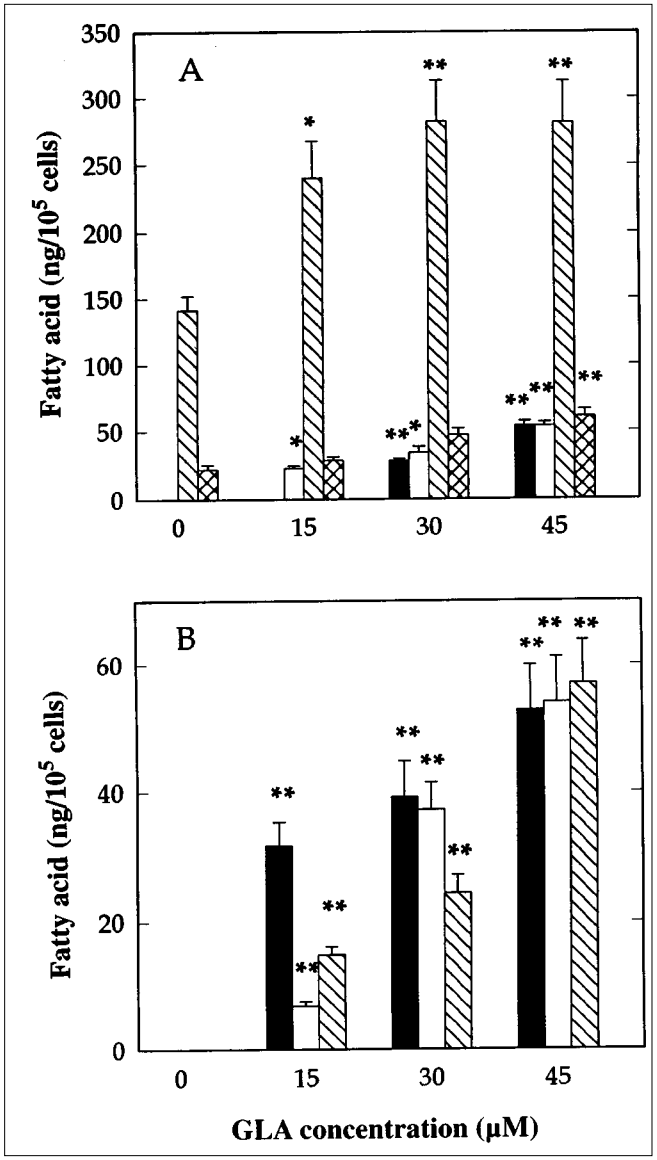

FIG. 1. Effect of 15–45 μM γ-linolenic acid (GLA) supplementation on the amounts of (■) GLA, (□) dihomo-γ-linolenic acid, (diagonally striped bar) arachidonic acid, and (cross-hatched bar) docosatetraenoic acid in (A) phospholipids and (B) neutral lipids of 36B10 cells. Cells were supplemented with GLA for 24 h. Values represent mean ± SE from four observations. *Significantly different from unsupplemented controls, $P < 0.05$; **significantly different from unsupplemented controls, $P < 0.01$.

compared with controls. DHA was not detected in any of these extracts.

The effect of 15–45 μM DHA supplementation on 36B10 cells is illustrated in Figure 3. Incubation of cells with 15–45 μM DHA increased the quantity of this fatty acid in the phospholipid and neutral lipid fractions. EPA and DPA were not detected in any of these cell extracts.

Cells incubated with 15–45 μM of OA showed a 10–25% increase in incorporation of this fatty acid into phospholipids and neutral lipids (data not shown). A decrease in the palmitoleic acid and linoleic acid content of phospholipids was also observed.

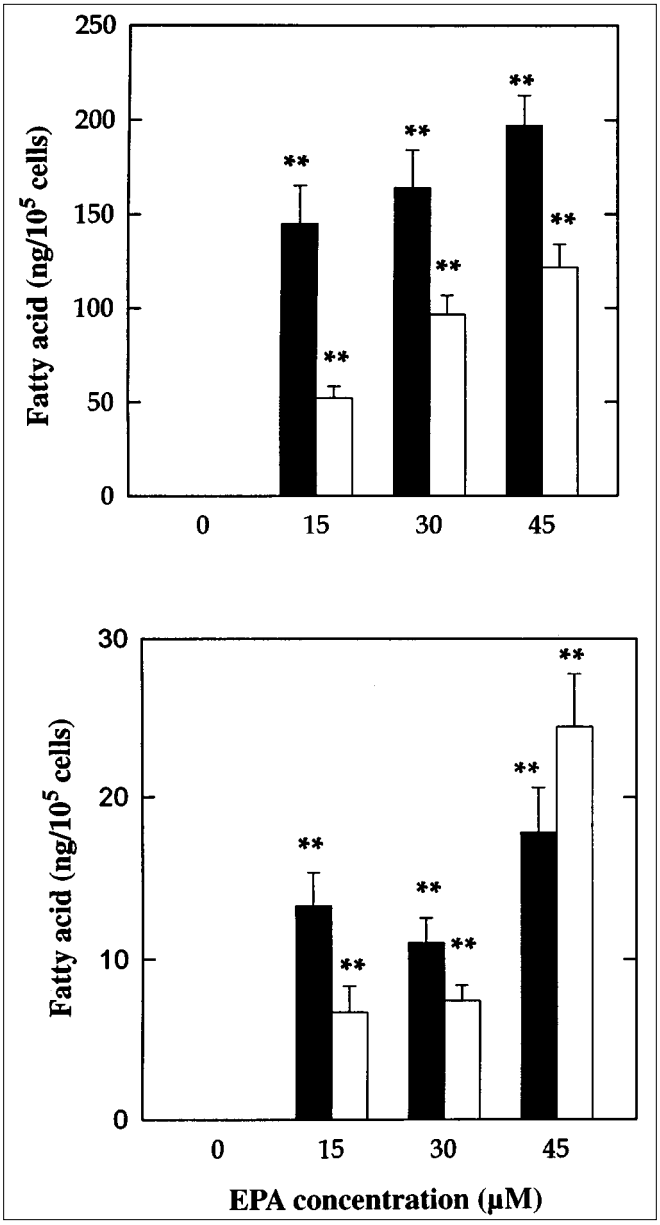

FIG. 2. Effect of 15–45 μM eicosapentaenoic acid (EPA) supplementation on the amounts of (■) EPA and (□) docosapentaenoic acid in (A) phospholipids and (B) neutral lipids of 36B10 cells. Cells were supplemented with EPA for 24 h. Values represent mean ± SE from four observations. **Significantly different from unsupplemented controls; $P < 0.01$.

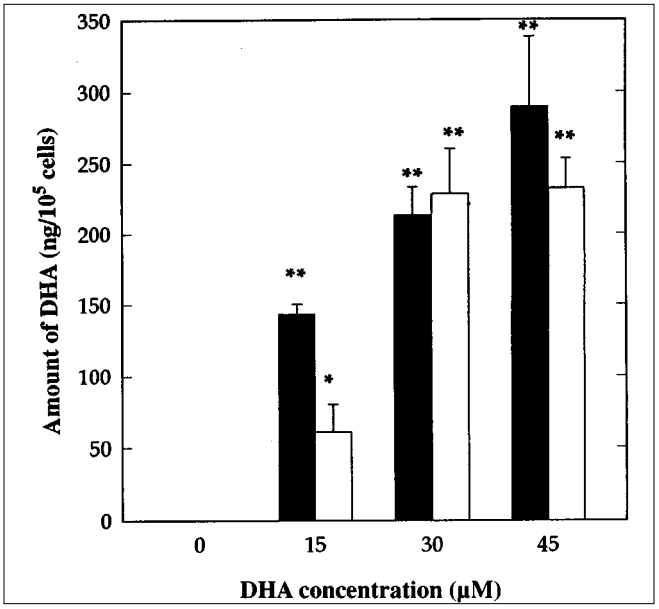

FIG. 3. Effect of 15–45 μM docosahexaenoic acid (DHA) supplementation on the amounts of DHA in the (■) phospholipids and the (□) neutral lipids of 36B10 cells. Cells were supplemented with DHA for 24 h. Values represent mean ± SE from four observations. *Significantly different from unsupplemented controls, $P < 0.05$; **significantly different from unsupplemented controls, $P < 0.01$.

Figure 4 shows the effects of GLA, EPA, DHA, and OA on the methylene bridge indices of phospholipids and neutral lipids of rat astrocytoma cells. GLA, EPA, and DHA supplementation resulted in a dose-dependent increase in the methylene bridge indices of phospholipids and neutral lipids (Fig. 4A, B and C). As observed in the case of total PUFA content and the fatty acid profiles, the extent of increase produced was greater in the neutral lipids. Further, the increases in methylene bridge index after fatty acid supplementation were similar for cells incubated with GLA or EPA (Fig. 4A and B). However, when the cells were incubated with 30 or 45 μM DHA, the methylene bridge indices were higher as compared with those obtained by supplementing the cells with similar concentrations of GLA or EPA (Fig. 4C). OA did not change the methylene bridge index of phospholipids or neutral lipids at any of the concentrations used (Fig. 4D).

Supplementation for 24 h with 15–45 μM GLA, EPA, or DHA produced only minor changes in the cholesterol and phospholipid content of cells (data not shown). Moreover, the cholesterol/phospholipid ratios did not change, except for cells supplemented with DHA, where a small decrease in cholesterol/phospholipid ratio was observed.

Effect of fatty acids on cell survival. Figure 5 shows that incubation of the 36B10 astrocytoma cells with GLA, EPA, and DHA throughout the experimental period reduced their clonogenic survival. Supplementing cells with 45 μM GLA and DHA decreased the surviving fraction of cells to 55.2 ± 8.8% and 64.8 ± 7.0% of the control value, respectively. EPA was extremely cytotoxic; supplementing with 15 μM EPA reduced the surviving fraction of cells to 25%. The cytotoxic effects of EPA increased in a dose-dependent fashion and at 45 μM EPA, no cells survived. OA was not cytotoxic at any of the concentrations used (Fig. 5).

Effect of fatty acids on the radiation response of 36B10 cells. The effect of supplementation of 36B10 cells with GLA, EPA, DHA, and OA prior to, during, and after irradiation on their response to radiation is shown in Figure 6. OA

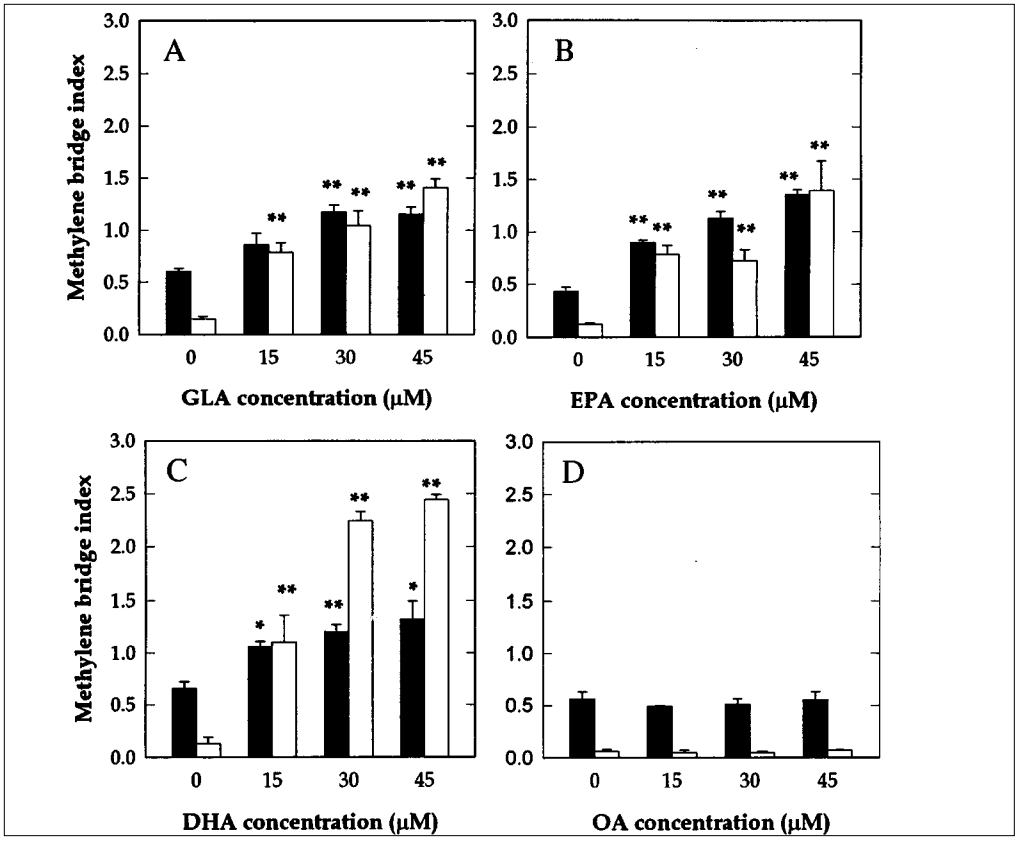

FIG. 4. Effect of (A) GLA, (B) EPA, (C) DHA, and (D) oleic acid (OA) supplementation on the methylene bridge index of the phospholipids (■) and neutral lipids (□) in 36B10 cells. Cells were supplemented with 15–45 μM of the fatty acid for 24 h. Values represent mean ± SE from four observations. *Significantly different from unsupplemented controls, $P < 0.05$; **significantly different from unsupplemented controls, $P < 0.01$. See Figures 1–3 for other abbreviations.

had no effect on survival when the cells were exposed to 0–10 Gy γ-rays (Fig. 6A). On the other hand, GLA (Fig. 6B), DHA (Fig. 6C), and EPA (Fig. 6D), when combined with radiation, led to a substantial increase in cell kill as compared with either radiation or these fatty acids alone. The increase in radiation-induced cell kill by EPA and DHA was significant at concentrations of 30 μM ($P < 0.05$; D_0 Gy = 1.97 ± 0.06) and DHA at 45 μM ($P < 0.01$; D_0 Gy = 2.26 ± 0.05), respectively. EPA was too cytotoxic at 45 μM to allow reliable estimates of additional effects of irradiation. GLA significantly enhanced the radiation response of the cells at concentrations ≥15 μM ($P < 0.01$). Thus, GLA was the most effective of these fatty acids in selectively increasing the sensitivity of cells to radiation across all dose levels. The mean (±SE) D_0 Gy value obtained for cells supplemented with GLA was significantly reduced ($P < 0.01$; D_0 Gy = 1.92 ± 0.04 for 45 μM GLA concentration) as compared with that of cells which received no fatty acid supplement (D_0 Gy = 2.70 ± 0.04).

Effect of the time of fatty acid supplementation on the radiation response of 36B10 cells. To determine whether supplementation with the fatty acids before, during, and after irradiation was essential for the cells to express a modified radiation response, the cells were incubated with fatty acid-supplemented media at different times prior to or after irradiation, and clonogenic survival was subsequently determined. Table 2 shows the effect of a single 24-h incubation of cells with GLA, followed by irradiation (5 Gy) immediately or at intervals of 24 or 48 h, on the survival of cells. None of the fatty acid concentrations, when supplemented for only 24 h, produced cytotoxic effects on their own. When cells were irradiated immediately after supplementation for 24 h with 15–45 μM GLA, they exhibited increased radiation-induced cell kill. However, with increasing interval between GLA supplementation and irradiation, the survival of the GLA-supplemented groups returned to control values. In similar experiments with EPA, DHA or OA, no increase in radiation induced cell kill was observed when the cells were incubated with these fatty acids only for the 24 h prior to irradiation (data not shown).

The radiation response of cells supplemented with 15–45 μM GLA or DHA after irradiation with 2.5–10 Gy γ-rays is shown in Figure 7. Addition of GLA (≥15 μM) or DHA (≥30

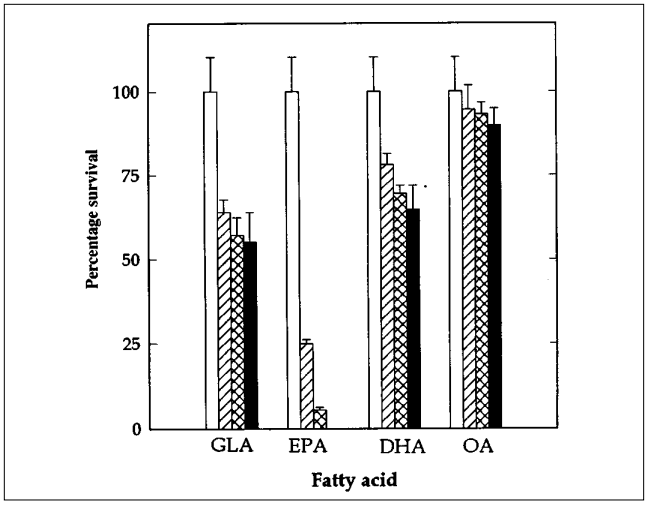

FIG. 5. Effect of GLA, EPA, DHA, and OA supplementation on the survival of 36B10 cells. Cells were supplemented with (diagonally striped bar) 15, (cross-hatched bar) 30, or (■) 45 μM of the fatty acid throughout the duration of the experiment. Control cells (□) received no supplemental fatty acid. Bars indicate values obtained from six separate cultures (mean ± SE). See Figures 1–4 for abbreviations.

μM) increased radiation-induced cell kill ($P \leq 0.01$). The enhanced cell kill increased with radiation dose and concentration of the fatty acid. The dose-response curves were similar to those obtained when the fatty acid was present before, during, and after irradiation (compare Fig. 6B and C with Fig. 7A and B). GLA and DHA also reduced the D_0 Gy values for the astrocytoma cells to 2.02 ± 0.04 ($P < 0.01$) and 2.05 ± 0.06 ($P < 0.01$), respectively (45 μM concentration), as compared with 2.70 ± 0.04 when the cells received no fatty acid supplement. In contrast, supplementation of 36B10 cells with EPA and OA after irradiation did not alter the radiation response of the cells (data not shown).

Effect of α-tocopherol acetate on the radiation response of GLA- and DHA-supplemented cells. α-Tocopherol acetate, on its own, did not affect the survival of the astrocytoma cells (data not shown). When added to GLA- or DHA-supplemented media however, it blocked the cytotoxic action of both the fatty acids (data not shown). Furthermore, the addition of 50 or 100 μM α-tocopherol acetate also blocked the enhanced radiation-induced cell kill caused by 45 μM GLA and DHA (Fig. 8).

TABLE 2
Effect of Time of Irradiation Following GLA Exposure on 36B10 Astrocytoma Cell Survival[a]

GLA concentration (μM)	Surviving fraction of cells		
	0 h[b]	24 h[b]	48 h[b]
Control	0.18 ± 0.015	0.18 ± 0.015	0.18 ± 0.015
15	0.13 ± 0.009*	0.17 ± 0.005	0.20 ± 0.028
30	0.11 ± 0.008*	0.14 ± 0.025	0.22 ± 0.018
45	0.09 ± 0.004**	0.11 ± 0.011*	0.19 ± 0.009

[a]Cells were supplemented for 24 h with 15–45 μM γ-linolenic acid (GLA) and returned to unsupplemented media. The cells were then irradiated at intervals of 0, 24, or 48 h thereafter with 5 Gy. Control cells received no GLA supplement. Each value represents mean ± SE from at least six separate cultures. All values have been normalized with respect to their respective unirradiated controls. *$P < 0.05$ and **$P < 0.01$ significantly different from the unsupplemented group.
[b]Interval in hours between 24 h GLA supplementation and 5 Gy irradiation.

DISCUSSION

The present results show that supplementation of astrocytoma cells with PUFA produces marked changes in the fatty acid composition of their phospholipids and neutral lipids. GLA, EPA, and DHA produce cytotoxic effects on the cells. Further, the radiation-induced cytotoxicity of these cells can be enhanced by GLA, EPA, or DHA supplementation.

Changes in fatty acid composition as a result of alterations in the type of fatty acid available have been reported *in vivo* and *in vitro* (15). However, most of these investigations have measured changes in fatty acid composition of total lipids or in phospholipids, and the effects on the cells have been mainly attributed to changes in membrane phospholipid composition (4). Our results show that in addition to altering the phospholipid content, PUFA supplementation produces marked changes in the fatty acid composition and methylene bridge index of the neutral lipids. In fact, the relative changes produced in neutral lipids are much greater than those produced in phospholipids. These results suggest that neutral lipid composition also might play a role in the effects of PUFA on tumor cell properties or their responsiveness to anticancer therapy.

Supplementation with each of the three PUFA produced some cytotoxicity, even without subsequent irradiation. This may be due to a lesser ability of astrocytoma cells to protect themselves against the level of free radical formation that occurs under basal conditions. Antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase are the primary mechanisms for scavenging reactive oxygen species. The activities of these antioxidant enzymes are decreased in many tumor cells (22–24). Tumor cells have a lower PUFA content than nonmalignant tissues (25,26), possibly as a compensation that protects against their sensitivity to free radicals. In addition, tumor cells often are also deficient in fatty acid Δ-6 desaturase, an enzyme that is required to further desaturate linoleic and α-linolenic acid to metabolic products such as GLA, AA, and EPA (27). The content of highly unsaturated fatty acids is low in these tumors as a result of this, probably accounting for the fact that tumor cells are reported to exhibit less lipid peroxidation than normal cells even though they are more inherently sensitive to oxidants (28,29). The PUFA in these cells is replaced by OA, which is not a highly reactive substrate for lipid peroxidation (30,31). Preliminary studies in our laboratory show that the OA content of normal rat astrocytes is 75% less than in the 36B10 astrocytoma when grown under similar conditions. Brain tumors also contain more OA and less PUFA than normal brain tissue (26)

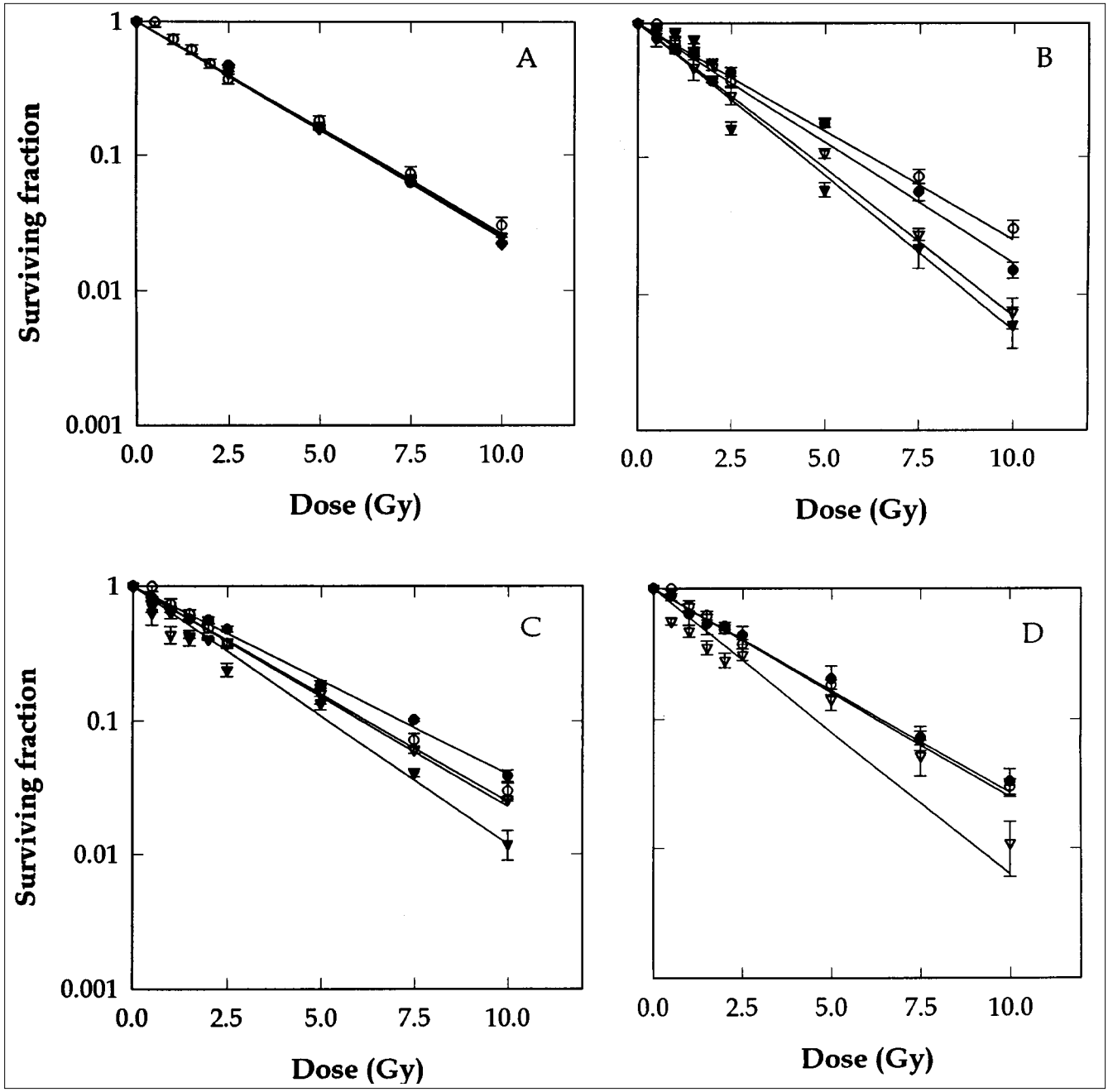

FIG. 6. Effect of (A) OA, (B) GLA, (C) DHA, and (D) EPA supplementation on the radiation response of 36B10 cells. Cells were supplemented with (●) 15, (▽) 30, or (▼) 45 μM of the fatty acid for 24 h prior to and during irradiation and were continued in the fatty acid-enriched media throughout the duration of the experiment. Control cells (○) received no supplemental fatty acid. The symbols indicate values obtained from six separate cultures (mean ± SE). The lines were drawn using scattered linear regression analysis. See Figures 1–4 for abbreviations.

Supplementation of the astrocytoma cells with GLA, EPA, or DHA provided these cells with an excess of substrate for lipid peroxidation. The lesser ability of the astrocytoma cells to neutralize the resulting increase in free radical formation in response to ordinary levels of oxidant stress may account for the PUFA-induced cytotoxicity seen in the absence of radiation. Indeed, providing the cells with the α-tocopherol acetate to increase membrane antioxidant defenses blocked the cytotoxic effects of GLA and DHA. This supports our hypothesis that the cytotoxic effect is in part due to increased oxidative stress resulting from PUFA supplementation. While GLA and DHA at the concentrations used produced a relatively small cytotoxic effect under basal conditions, EPA was extremely cytotoxic to the cells. The reason for the greater toxicity of EPA is not known and needs to be investigated. Further, although the increase in methylene bridge index was

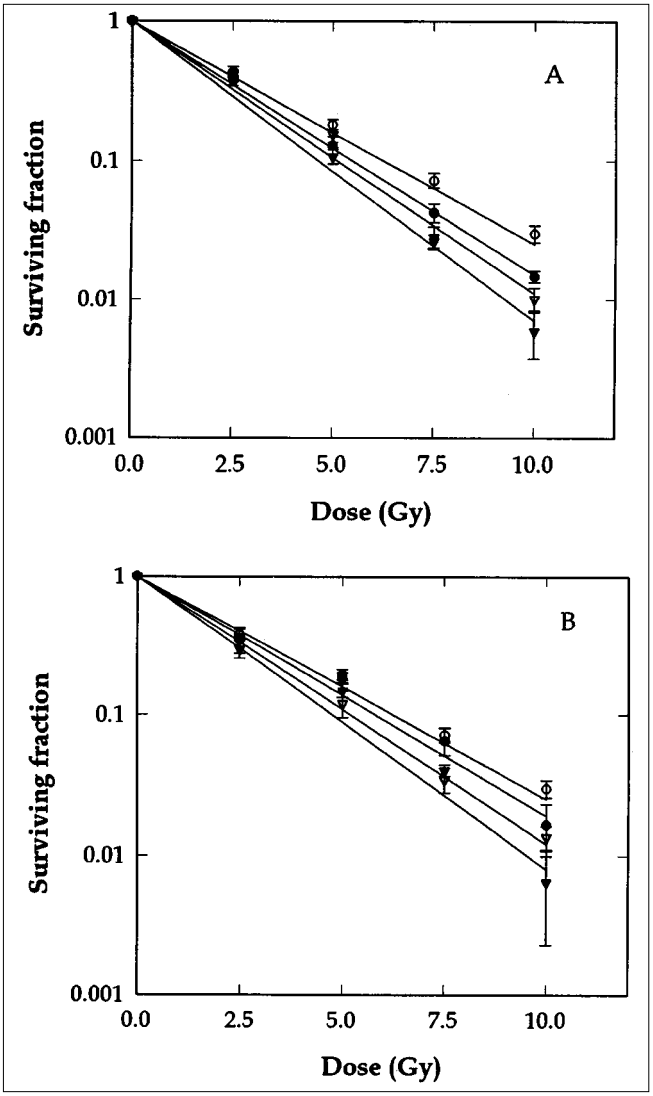

FIG. 7. Effect of (A) GLA, and (B) DHA supplementation post-irradiation on the radiation response of 36B10 cells. Cells were supplemented with (●) 15, (▽) 30, or (▼) 45 μM of the GLA or DHA within 1 h following irradiation and were incubated in the fatty acid-enriched media throughout the remainder of the experiment. Control cultures, ○, were not exposed to supplemental fatty acid. The symbols indicate values obtained from six separate cultures (mean ± SE). The lines were drawn using scattered linear regression analysis. See Figures 1 and 3 for abbreviations.

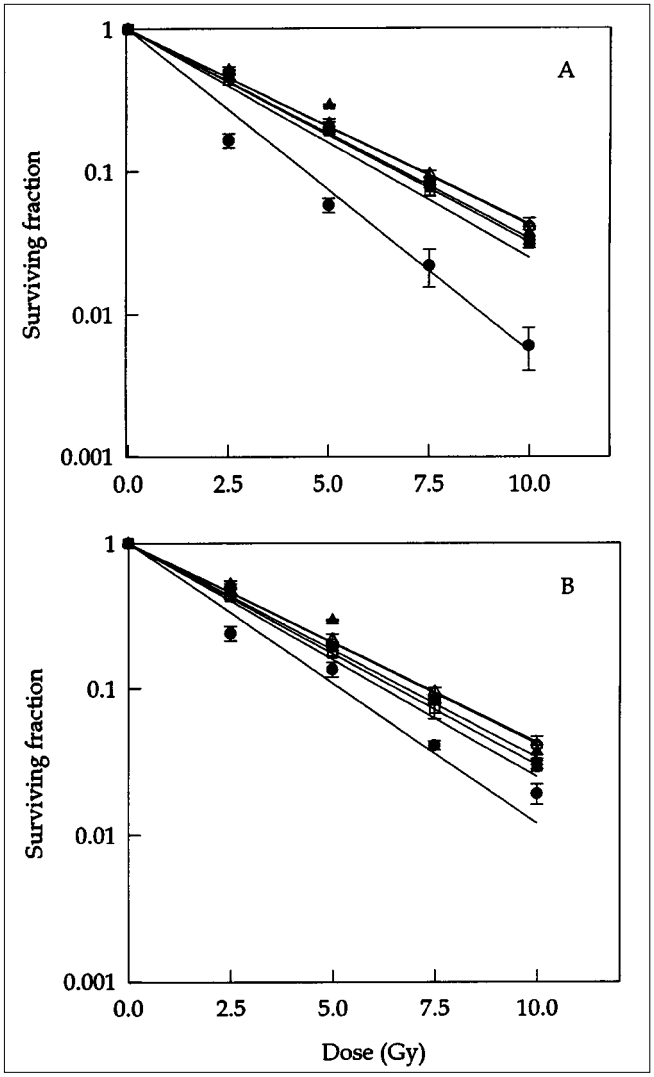

FIG. 8. Effect of α-tocopherol acetate on the radiation response of 36B10 cells enriched with (A) GLA, and (B) DHA. Cells were incubated in 50 or 100 μM α-tocopherol acetate with or without 45 μM GLA or DHA. (○) Controls, (●) 45 μM GLA or DHA, (▽) 50 μM α-tocopherol acetate, (▼) 100 μM α-tocopherol acetate, (□) 50 μM α-tocopherol acetate + GLA or DHA, (■) 100 μM α-tocopherol acetate + GLA or DHA. The symbols indicate values obtained from six separate cultures (mean ± SE). The lines were drawn using scattered linear regression analysis. See Figures 1 and 3 for abbreviations.

greater for cells supplemented with DHA, this fatty acid was only moderately cytotoxic. These results suggest that the cytotoxic effects of PUFA may not be the result of a single straightforward mechanism such as membrane disruption due to lipid peroxidation, but may also involve other mechanisms such as generation of biologically active fatty acid oxidation products.

DHA increased the radiation response of the astrocytoma cells only at a concentration of 45 μM. Although EPA increased the radiation response of cells at 30 μM, this fatty acid on its own was extremely cytotoxic. Data on the clonogenic cell survival assay using EPA alone yielded very few colonies. Therefore, the results using EPA and radiation must be interpreted with caution; without including EPA, GLA was most effective in increasing the responsiveness of the astrocytoma cells to radiation at concentrations ≥15 μM. There have been several reports describing antitumor effects of GLA (32-33). The cytotoxic effect of GLA in tumor cells is accompanied by increased thiobarbituric acid-reactive substances and thought to be mainly the result of increased generation of free radicals and lipid peroxidation (33,34). Free radical release and lipid peroxidation in cells is further aug-

mented when PUFA-supplemented cells are exposed to an oxidant such as iron (35,36).The enhancement in radiation-induced cell kill by GLA observed by us could be the result of radiation, like iron, acting as an oxidant which induces free radical formation and lipid peroxidation.

An important aspect of the antitumor effects of PUFA is that PUFA cause little or no damage to normal cells. *In vivo* studies on modulation of radiation-induced skin (37,38) and central nervous system (39) injury show that dietary PUFA significantly decrease the severity of radiation-induced injury to these normal tissues. Further, preliminary *in vitro* studies in our laboratory using GLA and DHA suggest that these PUFA on their own, or when combined with radiation, exert no deleterious effects on normal rat astrocytes. This suggests that GLA and DHA might selectively increase the radiation response of astrocytomas without causing measurable normal brain toxicity, a finding that has potential therapeutic implications.

In summary, supplementation of cells with 15–45 μM GLA, EPA, or DHA produces significant alterations in the lipid composition of 36B10 rat astrocytoma cells. *In vivo* studies also indicate that dietary supplementation with PUFA can alter PUFA composition of not only normal brain tissue (40,41) but also that of intracerebrally implanted tumor tissue (42). Our results indicate that such alterations in fatty acid composition of brain tumors might make these tumors more susceptible to therapy with radiation. This suggests that PUFA may be potential tools for improving the efficacy of radiotherapy for certain malignant brain tumors.

ACKNOWLEDGMENTS

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Incorporation of Long-Chain n-3 Fatty Acids in Tissues and Enhanced Bone Marrow Cellularity with Docosahexaenoic Acid Feeding in Post-Weanling Fischer 344 Rats

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ABSTRACT: We wanted to examine the effects of an oil rich in docosahexaenoic acid (DHA), without eicosapentaenoic acid, on the composition of membrane phospholipid in a variety of tissues. Our *in vitro* studies had previously shown that DHA could modify glucose and nucleoside transport in cells in culture and also increase selectivity of the nucleoside drug, arabinosylcytosine (araC) toward tumor cells. Here we wanted to examine what effect DHA supplementation would have in the whole animal in terms of the chemosensitivity of normal bone marrow, the dose-limiting tissue during chemotherapy, to araC. The purpose was to determine whether fatty acid supplementation might be useful as an adjuvant to chemotherapy. We fed diets containing 5% (w/w) low fat–corn oil (LF–CO group), 10% moderate fat–safflower oil (MF–SO group), or 10% DHASCO™ (MF–DHA group) to weanling Fischer 344 rats for 8–9 wk. Feed intake and growth were not different between the different diets. Similarly, treatment of animals with the chemotherapeutic drug araC did not differentially affect growth, feed intake, or tissue fatty acid composition for the different diet groups. Fatty acid compositions of bone marrow, liver, red blood cells, plasma phospholipid and triglyceride, as well as skeletal and cardiac muscle, were substantially different between the dietary groups. The DHASCO™ oil contained 46%DHA (22:6n-3) and resulted in profound incorporation of DHA in all tissues examined. The most dramatic response was seen in skeletal muscle of MF–DHA fed animals where DHA represented 46% of membrane phospholipid fatty acids. This is likely to have consequences to muscle function. Although DHASCO™ contains a similar level of saturated fatty acids (42%), few differences in saturates were noted between the various dietary groups for most of the tissues examined. Both LF–CO and MF–SO diets were hypercholesterolemic, and the LF–CO was also hypertriglyceridemic compared to the chow-fed animals. Animals fed the MF–DHA diet had the lowest triglyceride levels of any of the treatment groups and cholesterol levels comparable to chow-fed animals. MF–DHA had substantially higher numbers of colony-forming units–granulocyte macrophage (CFU–GM) as reflected in a twofold higher bone marrow cellularity than either chow or LF–CO animals, suggesting expansion of the bone marrow compartment with DHA feeding. Although higher than LF–SO, the number of CFU–GM in MF–SO animals was not significantly higher than animals fed chow. Bone marrow from LF–CO animals appeared to be more resistant to araC treatment than either MF group. Thus, DHA, fed as DHASCO™, has advantages over low or moderate n-6 diets and chow as it is has both hypolipidemic- and bone marrow-enhancing properties in weanling Fischer 344 rats. This suggests that DHA supplementation may be useful in adjuvant chemotherapy.

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Populations that consume a high proportion of fish and aquatic mammals such as Greenland Inuit and Japanese tend to have lower incidence of a number of diseases including coronary artery disease and mammary and colon cancers (1, for review see 2). The long-chain ω3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) have been suggested to be the major dietary constituents responsible for reducing the incidence of chronic disease. Recently, intervention trials with fish oil supplements have shown hypotriglyceridemic effects in hyperlipidemic subjects and reduced the incidence and progression of tumors in a number of animal models, human cell lines, and in one study of human metastasis (3–10). The mechanisms by which ω3 fatty acids achieve their putative protective activity in cardiovascular disease development are being intensively investigated and presumably include their activity as hypotriglyceridemic agents and in altering the composition and number of lipoproteins in the circulation. The ω3 fatty acids can also modulate the production of eicosanoids and prostaglandins (reviewed in 11) influencing immune responses and cellular growth and differentiation processes critical to cardiovascular disease and tumor development. Purified EPA has been tested in animal studies (12,13), and on some human cell lines (14,15), but there are

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Abbreviations: AA, arachidonic acid; araC, arabinosylcytosine; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DTA, docosatetraenoic acid; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; IMDM, Iscove's Modified Dulbecco's Medium; LA, linoleic acid; LF–CO, low fat corn oil; MF–SO, moderate fat safflower oil; OA, oleic acid; PBS; phosphate buffered saline; PUFA, polyunsaturated fatty acids.

few reports on the specific action of purified DHA on adolescent animal growth, as a hypolipidemic agent or as a cancer-preventive agent. Neither has the possible use of DHA as an adjuvant chemotherapeutic agent been extensively studied in clinical or experimental animal models.

We recently demonstrated that dietary fish oil results in decreased rates of uptake of nucleosides into human erythrocytes and this correlated with changes in the fatty acid composition of membrane phospholipids in these cells (16). Our *in vitro* results comparing murine leukemia cells to normal bone marrow-derived macrophages showed that both DHA and EPA sensitized L1210 leukemia cells to the nucleoside drug arabinosylcytidine (araC) and the anthracycline drug adriamycin (17). In another study, we reported that purified DHA inhibited growth of transformed rat-2 fibroblasts in culture and additionally protected normal rat-2 fibroblasts from araC (18). The n-6 fatty acids linoleic and linolenic acids had no such activity. The current trial set out to determine whether changes in drug toxicity observed *in vitro* using purified DHA, would be mimicked in whole animal feeding of a DHA (EPA-free) diet. In our current investigation, weanling Fischer 344 rats were fed a 5% n-6 fat, 10% n-6 fat, or DHA-rich diet (10% fat) for two months. One week before the end of the trial, one-half of the animals were treated with a single six-day course of araC. Toxicity indices, including hair loss, diarrhea, and bone marrow suppression, were then compared between dietary treatment groups. Because DHA feeding in the absence of EPA has not been extensively examined, our study design also allowed us to assess the hypolipidemic effects of DHA directly and to examine specific tissue distribution of this fatty acid and its effects on overall fatty acid composition of a variety of tissues.

MATERIALS AND METHODS

Materials. Weanling male Fischer rats were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Diet ingredients were purchased from the following sources: DHASCO™ (a gift from Martek Biosciences Corp., Columbia, MD), corn oil (Mazola, local distributor), safflower oil (President's Choice, local distributor), minerals (BDH, Pool, England, and Fisher, Fairlawn, NJ), ferric citrate (Sigma, St. Louis, MO), cornstarch (Casco, Etobicoke, Ontario, Canada), casein (ICN, Aurora, OH), and all other diet components (United States Biochemicals, Cleveland, OH). Cholesterol (procedure 352) and triglyceride (procedure 339) quantitation kits were obtained from Sigma Chemical Co.

Animal care and feeding. Three-week-old male Fischer rats were received, weighed, and acclimated on Purina rat chow for 3 d. Animals were housed individually in wire-bottom cages and provided with 15-cm diameter plastic tubing for environmental enrichment. Animals were maintained in a climate-controlled animal facility with 12-h light/12-h dark cycle. Procedures were approved by the University of Guelph, Animal Welfare Committee, in accordance with the Guidelines from the Canadian Council on Animal Care. On experimental day 0, animals were weighed and randomly assigned to dietary treatment groups (16–18 animals/group). An additional group of 11 animals was fed Purina rat chow *ad libitum.* Dietary treatment groups were designated: low fat–corn oil (LF–CO), moderate fat–safflower oil rich (MF–SO), and moderate fat–docosahexaenoic acid rich (MF–DHA). The energy composition of the LF–CO, MF–SO, and MF–DHA and chow diets were 11.7, 25.6, 25.6, and 11.3% of energy as fat, 67.6, 53.7, 53.7 and 62.9% of energy as carbohydrate, for each diet, respectively. All semipurified diets contained 20.7% of energy from protein with a total energy content of 16.2 MJ/kg diet. Chow contained 25.8% of energy from protein and a total energy content of 14.9 MJ/kg diet. The complete composition of the semipurified diets is shown in Table 1. Diets were prepared weekly by gassing with nitrogen and vacuum packing and were then stored at –20°C. Oxidation status of feeds was estimated by the TBARS assay and were well below acceptable limits (19) for CO, 0.4; MF–SO, 0.4; MF–DHA, 1.1 nmol malondialdehyde equivalents/g diet.

Animals were allowed free access to diets, and intake and weight gain were measured frequently over an 8-wk period. Feed was replaced daily. Seven days before the end of the study, half of the animals in each diet (except chow) were assigned to receive araC while the other half of the animals received saline injections (Therefore final animal numbers were 8–9 per group). Animals were injected once per day for six

TABLE 1
Diet Composition[a]

Component	LF–CO	MF–SO	MF–DHA
Casein	200	200	200
DL methionine	3	3	3
Choline bitartrate	2	2	2
Vitamin mix[b]	10	10	10
Mineral mix[c]	35	35	35
Fiber-cellufil	50	125	125
Sucrose	100	100	100
Corn starch	550	415	415
Corn oil	50	10	10
Safflower oil	—	100	—
DHASCO™[d]	—	—	100

[a]Grams per kilogram diet; MF–DHA, moderate fat–docosahexaenoic acid; LF–CO, low fat–corn oil; MF–SO, MF–safflower oil.
[b]Vitamin mix contains the following ingredients per kg mixture: 600 mg thiamin-HCl, 600 mg riboflavin, 700 mg pyridoxine-HCl, 3 g nicotinic acid, 1.6 g D-calcium pantothenate, 200 mg folic acid, 20 mg D-biotin, 1 mg cyanocobalamin, 400,000 IU vitamin A (800 mg retinyl palmitate), 5,000 IU vitamin E (5 g dl-α-tocopherol acetate), 2.5 mg cholecalciferol, 5.0 mg menaquinone, sucrose finely powdered to make up to 1000 g.
[c]Mineral mix contains the following ingredients per kg mixture: 500 g calcium phosphate dibasic, 74 g sodium chloride, 220 g potassium citrate monohydrate, 52 g potassium sulfate, 24 g magnesium oxide, 3.5 g manganous carbonate, 6 g ferric citrate, 1.6 g zinc carbonate, 0.3 g cupric carbonate, 0.01 g potassium iodate, 0.01 g sodium selenite, 0.55 g chromium potassium sulfate and the remaining mixture was made up to 1000 g with sucrose.
[d]The composition of the DHASCO™ was as follows: 10:0, 0.8%; 12:0, 6.2%; 14:0, 19.1%; 16:0, 16.1%; 16:1, 1.7%; 18:0, 0.3%; 18:1, 10.3%; 18:2n-6, 0.5%; 22:6n-3, 44.6%. Antioxidants in the oil were 0.005% tocopherol and 0.025% ascorbyl palmitate.

days subcutaneously with 50 mg/kg araC or an equivalent volume of saline solution. The injection sites were rotated between left and right flank each day. At the end of the experimental period, animals were drug-free for 24 h, fasted for 12 h, euthanized using metophane anesthetic followed by cardiac puncture and decapitation. An aliquot of the blood collected by cardiac puncture was frozen for future analysis or placed in tubes containing 0.129 M buffered sodium citrate (Vacutainer tubes; Becton Dickinson #6419, Franklin Lakes, NJ) and centrifuged to separate red cells and plasma and stored at −20°C.

Immediately after decapitation samples of liver, skeletal muscle (red quadriceps) and heart were recovered and quick frozen in liquid nitrogen. Tissues were stored in liquid nitrogen until they could be analyzed.

Bone marrow preparation and CFU-GM assay. Immediately after decapitation, femurs were recovered from the hind limbs and two–three aliquots (5 mL each) of Iscove's Modified Dulbecco's Medium (IMDM) containing 50 units/mL streptomycin and penicillin, 100 µg/mL gentamycin (Gibco-BRL, Burlington, Ontario, Canada) and 15% fetal bovine serum (FBS, Gibco-BRL) was flushed through (syringe and an 18-gauge needle) the marrow cavity to dislodge the marrow into a sterile 15-mL tube. Complete (at least 95%) removal of marrow from the femur was confirmed by visual and microscopic examination. Cells were dissociated by rapidly pipetting up and down and an aliquot counted using a Coulter Counter (Model ZM; Coulter Electronics, Burlington, Ontario, Canada) equipped with a channelizer. Confirmation that a single cell suspension was achieved was confirmed using a hemocytometer and microscope. Using the channelizer, at least three populations of cells could be identified by their differing size distribution profiles. Cells were washed twice in complete medium, counted and plated at 5×10^5 cells per dish (60 mm culture dish containing 5 mL) in 0.8% methylcellulose containing 20% FBS, antibiotics (as above), 10% WEHI-3 conditioned medium (containing the growth factors interleukin-3 and granulocyte–macrophage colony stimulating factor) and 10% L-cell conditioned medium (containing Macrophage colony stimulating factor at final concentration of 2000 units/mL). Seven days later the number of colonies in each of triplicate dishes for each animal was counted using an inverted light microscope. Colonies were scored blind using a cutoff of 16 cells/colony. The remaining bone marrow cells not plated were washed twice with IMDM and frozen in liquid nitrogen at 5×10^7 cells/mL in IMDM.

Fatty acid and lipid analyses. Bone marrow cells that had been washed a total of four times (two times in PBS and two times in IMDM) were frozen at the time animals were euthanized. Red cells were prepared by spinning in a clinical benchtop centrifuge and removing plasma. On one sample of plasma, total triglyceride (procedure #339; Sigma) and cholesterol (procedure #352; Sigma) estimates for all animals were made according to manufacturer's instructions. Red cells were washed three times in PBS, and the pellet frozen in liquid nitrogen. Frozen tissues or bone marrow and red blood cell samples were thawed, and total lipid was extracted according to the method of Bligh and Dyer (20). The phospholipid and triglyceride fractions were separated from other lipids by thin-layer chromatography on Silica Gel G Redi/Plates® (Fisher, Unionville, Ontario, Canada) developed with heptane/isopropyl ether/acetic acid (60:40:3). The origin, containing phospholipids, and the triglyceride fraction were scraped after visualization with 0.1% aminonaptholsulfonic acid and after elution from the cellulose, 3 µg 17:0 standard was added to the eluent and fatty acids methylated by adding 3 mL of methylating reagent (94% vol/vol methanol, 6% vol/vol sulfuric acid) and incubation at 80°C for 2 h. Ether extracts of fatty acids were prepared, ether removed, and fatty acids dissolved in carbon disulfide. Methylated fatty acids were analyzed by gas phase chromatography (21) using a 30-meter DB225 megabore column (J&W Scientific Inc., Folson, CA). Peaks were identified by comparison with a nerve standard containing fatty acids from 12 to 24 carbons in length.

RESULTS

Weanling Fischer 344 rats were acclimated for 3 d on Purina rat chow before being switched to one of the three experimental diets. Over the 8–9-wk feeding period, there were no differences in the consumption or growth patterns on any of the experimental diets (data not shown). During the araC drug treatment phase (the last six days of the experimental protocol), there was very modest weight loss that was not significantly different between diets (6.2, 4.3, and 7.6 g for LF–CO, MF–SO, and MF-DHA, respectively). We observed only minimal hair loss from araC-treated animals and none of the animals developed diarrhea or substantive anorexia.

We examined the plasma cholesterol and triglyceride levels from all three dietary groups including saline- and araC-treated animals as well as from control chow-fed rats. Drug treatment had no effect on either cholesterol or triglyceride levels (Table 2). Both moderate fat (25.6% of calories from fat) diets resulted in substantially lower total cholesterol and triglyceride levels than in the LF diet (11.7% of calories from fat). The MF–SO group had plasma cholesterol levels 19% lower than the LF–CO group. MF–DHA rats had cholesterol levels 48% lower than the LF–CO group and 41% lower than the MF–SO group. Chow-fed animals had cholesterol values essentially identical to those in DHA-fed animals and lower than both n-6 groups. The MF diets produced triglyceride levels that were 44% (MF–SO) and 49% (MF–DHA) lower than the LF–CO group. On the other hand, chow-fed animals had intermediate triglyceride levels that were not significantly different from any of the experimental diets. Therefore, both the safflower- and DHA-rich diets, despite being quite high in fat, still resulted in substantial hypolipidemia when compared to the LF–CO diet and gave similar lipemic responses to those seen in chow fed (4.5% dietary fat) animals.

Both plasma phospholipid and triglyceride fatty acid compositions were remarkably susceptible to our dietary manipulations (Table 3). In particular arachidonic acid (AA, 20:4n-6) was decreased by 92% by the MF–DHA diet rela-

TABLE 2
Cholesterol and TG Levels in Plasma from Rats Fed the Experimental Diets[a]

	LF–CO saline ($n = 7$)	LF–CO araC ($n = 7$)	MF–SO saline ($n = 9$)	MF–SO araC ($n = 9$)	MF–DHA saline ($n = 9$)	MF–DHA araC ($n = 7$)	Chow ($n = 11$)
Chol (mg/dl)	105 ± 9^{c}	96 ± 4^{c}	75 ± 7^{b}	78 ± 7^{b}	44 ± 4^{a}	49 ± 4^{a}	44 ± 2^{a}
TG mg/dl)	$172 \pm 16^{b,c}$	198 ± 24^{c}	114 ± 13^{a}	111 ± 17^{a}	107 ± 10^{a}	95 ± 15^{a}	$137 \pm 7^{a,b}$

[a]Chol, cholesterol; TG, triglyceride; values represent means ± the standard error of the mean. Values not sharing a superscript within a row are statistically different by analysis of variance followed by Student-Newman Keuls, $P < 0.05$. See Table 1 for abbreviations.

tive to the LF–CO and MF–SO diets. Docosatetraenoic acid (DTA, 22:4n-6) was decreased to undetectable levels and docosapentaenoic acid (DPA, 22:5n-6) by 90% in MF–DHA-fed animals. Predictably, DHA levels were 5–8-fold higher in the phospholipid fraction from MF–DHA animals compared to both the LF–CO and MF–CO groups. EPA levels were similarly affected. Because there is no detectable EPA in the DHASCO™ oil, this latter observation suggests considerable retroconversion of DHA back to EPA.

The fatty acid composition of plasma triglyceride was affected in a similar fashion to plasma phospholipid (Table 3). We observed a higher level of linoleic acid (LA, 18:2n-6) in the MF–SO group compared to the LF–CO group. This difference may be due to the different content of LA in CO (61%) compared to SO (78%). LA levels were lowest in the MF–DHA rats. AA levels were affected in a similar manner to LA levels, highest in MF–SO, intermediate in LF–CO, and lowest in MF–DHA fed animals. Changes in triglyceride EPA, DPA, and DHA were analogous to those seen in the phospholipid fraction of plasma.

Of all of the tissues examined, skeletal muscle and heart appeared to be the most profoundly affected in terms of in-

TABLE 3
Fatty Acid Analysis of Tissues—Plasma Phospholipid and Serum Triglyceride[a]

	Plasma phospholipid			Serum triglyceride		
	Corn oil ($n = 6$)	Safflower oil ($n = 6$)	DHASCO ($n = 6$)	Corn oil ($n = 6$)	Safflower oil ($n = 6$)	DHASCO ($n = 6$)
14:0	0.2 ± 0.04^{a}	0.2 ± 0.05^{a}	0.6 ± 0.05^{b}	0.6 ± 0.02^{a}	0.4 ± 0.04^{a}	1.1 ± 0.07^{b}
16:0	21.3 ± 0.82^{a}	21.2 ± 0.71^{a}	28.9 ± 0.42^{b}	25.6 ± 0.55^{a}	21.6 ± 0.61^{b}	24.9 ± 0.83^{a}
16:1	0.2 ± 0.04	0.1 ± 0.03	0.2 ± 0.04	1.8 ± 0.10^{a}	0.6 ± 0.11^{b}	1.4 ± 0.38^{a}
18:0	25.0 ± 0.70^{a}	26.3 ± 0.60^{a}	21.1 ± 0.49^{b}	1.9 ± 0.12^{a}	2.4 ± 0.06^{ab}	3.1 ± 0.41^{b}
18:1	6.9 ± 0.47^{a}	4.9 ± 0.48^{b}	7.8 ± 0.21^{a}	24.1 ± 0.66^{a}	14.0 ± 0.86^{b}	18.4 ± 0.67^{c}
18:2n-6	8.0 ± 0.16^{a}	10.0 ± 0.33^{b}	10.6 ± 0.17^{b}	28.1 ± 1.23^{a}	37.6 ± 1.02^{b}	11.1 ± 1.12^{c}
18:3n-6	n.d.	n.d.	n.d.	0.3 ± 0.08^{a}	0.6 ± 0.12^{b}	n.d.[c]
20:1	0.4 ± 0.02^{a}	0.4 ± 0.03^{b}	0.3 ± 0.00^{b}	0.6 ± 0.07	0.5 ± 0.06	0.3 ± 0.09
20:2n-6	0.2 ± 0.00^{a}	0.5 ± 0.08^{b}	0.1 ± 0.00^{a}	0.3 ± 0.08^{a}	0.5 ± 0.06^{b}	n.d.[c]
20:3n-6	0.1 ± 0.02^{a}	0.1 ± 0.03^{a}	0.6 ± 0.02^{b}	0.1 ± 0.00	0.2 ± 0.08	0.1 ± 0.00
20:4n-6	25.0 ± 0.57^{a}	22.7 ± 0.70^{b}	1.9 ± 0.20^{c}	10.5 ± 0.52^{a}	14.5 ± 0.33^{b}	1.1 ± 0.46^{c}
20:5n-3	n.d.[a]	n.d.[a]	4.2 ± 0.16^{b}	0.1 ± 0.00^{a}	0.1 ± 0.02^{a}	6.1 ± 0.39^{b}
22:0	0.5 ± 0.05	0.7 ± 0.04	0.5 ± 0.07	0.1 ± 0.00	0.1 ± 0.02	0.1 ± 0.02
22:4n-6	0.9 ± 0.04^{a}	1.5 ± 0.09^{b}	n.d.[c]	2.4 ± 0.16^{a}	3.2 ± 0.22^{b}	0.3 ± 0.10^{c}
22:5n-6	4.5 ± 0.36^{a}	5.7 ± 0.42^{b}	0.5 ± 0.10^{c}	1.8 ± 0.10^{a}	2.3 ± 0.17^{b}	n.d.[c]
22:5n-3	0.1 ± 0.06	0.1 ± 0.02	n.d.	0.2 ± 0.02^{a}	0.1 ± 0.02^{a}	2.1 ± 0.93^{b}
22:6n-3	3.4 ± 0.18^{a}	2.3 ± 0.21^{a}	19.3 ± 0.72^{b}	0.6 ± 0.06^{a}	0.4 ± 0.06^{a}	28.6 ± 2.17^{b}
24:0	1.6 ± 0.23	1.7 ± 0.11	1.5 ± 0.12	0.1 ± 0.02^{a}	0.1 ± 0.02^{a}	n.d.[b]
24:1	1.0 ± 0.27	1.1 ± 0.24	0.5 ± 0.19	0.1 ± 0.02^{a}	n.d.[b]	n.d.[b]
Saturates	48.9 ± 1.9	50.5 ± 1.5	53.0 ± 1.2	25.5 ± 0.16	23.6 ± 0.1	26.6 ± 1.1
MUFA	8.6 ± 0.4^{a}	6.7 ± 0.8^{b}	9.0 ± 0.5^{a}	26.5 ± 0.9^{a}	15.2 ± 1.1^{b}	20.1 ± 1.1^{c}
PUFA	42.5 ± 1.0^{a}	43.2 ± 1.0^{a}	37.5 ± 1.0^{b}	44.8 ± 2.0^{a}	60.0 ± 1.4^{b}	51.2 ± 4.6^{a}
n-6	38.8 ± 0.8^{a}	40.6 ± 0.8^{a}	13.7 ± 0.3^{b}	43.5 ± 2.0^{a}	59.1 ± 1.3^{b}	14.0 ± 1.9^{c}
n-3	3.7 ± 0.2^{a}	2.6 ± 0.2^{a}	23.8 ± 0.7^{b}	1.2 ± 0.0^{a}	0.8 ± 0.1^{a}	37.1 ± 2.7^{b}
n-6/n-3	10.8 ± 0.5^{a}	16.2 ± 1.6^{b}	0.6 ± 0.0^{c}	37.9 ± 2.8^{a}	83.9 ± 10.5	0.4 ± 0.1^{c}

[a]Following termination of the feeding trial tissues were excised and quick frozen in liquid nitrogen. Lipids were extracted as described in the Materials and Methods section. For all tissues, the fatty acid composition as a percentage of total fatty acids in the phospholipid fraction is given. For serum, the fatty acid composition of both the phospholipid and triglyceride fractions was examined. Values represent means ± the standard errror of the mean. Numbers of animals examined is given in brackets. Values in a row not sharing a superscript are significantly different by analysis of variance followed by Student-Newman-Keuls post-test, $P < 0.05$; n.d. = not detected.

TABLE 4
Fatty Acid Analysis of Tissues—Skeletal Muscle and Heart[a]

	Skeletal muscle			Heart		
	Corn oil (n = 6)	Safflower oil (n = 6)	DHASCO (n = 6)	Corn oil (n = 6)	Safflower oil (n = 6)	DHASCO (n = 6)
14:0	0.2 ± 0.02[a]	0.1 ± 0.02[b]	0.7 ± 0.02[c]	0.1 ± 0.00[a]	0.1 ± 0.03[a]	0.3 ± 0.02[b]
14:1	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.02[a]	n.d.[b]	n.d.[b]
16:0	22.5 ± 0.55[ab]	21.2 ± 1.07[a]	24.5 ± 0.59[b]	11.0 ± 0.27[a]	9.4 ± 0.17[b]	12.0 ± 0.34[c]
16:1	0.3 ± 0.03[a]	0.1 ± 0.00[b]	0.3 ± 0.04[a]	0.1 ± 0.02	0.1 ± 0.00	0.1 ± 0.02
18:0	14.1 ± 0.32[a]	14.7 ± 0.70[a]	11.2 ± 0.16[b]	25.7 ± 0.25[a]	27.2 ± 0.19[b]	25.6 ± 0.61[a]
18:1	7.5 ± 0.15[a]	5.6 ± 0.17[b]	5.5 ± 0.11[b]	5.3 ± 0.09[a]	3.3 ± 0.06[b]	3.3 ± 0.14[b]
18:2n-6	11.7 ± 0.70[a]	13.0 ± 1.12[a]	8.7 ± 0.22[b]	12.1 ± 0.40[a]	12.2 ± 0.36[a]	8.0 ± 0.16[b]
18:3n-6	n.d.	n.d.	n.d.	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00
20:1	0.2 ± 0.02[a]	0.1 ± 0.02[b]	0.1 ± 0.00[b]	0.2 ± 0.02[a]	0.2 ± 0.00[a]	0.3 ± 0.02[b]
20:2n-6	0.2 ± 0.00[a]	0.4 ± 0.02[b]	n.d.[c]	0.2 ± 0.02[a]	0.3 ± 0.00[b]	0.1 ± 0.00[c]
20:3n-6	n.d.	n.d.	0.2 ± 0.00	n.d.[a]	n.d.[a]	0.3 ± 0.02[b]
20:4n-6	22.6 ± 0.26[a]	22.9 ± 0.85[a]	1.6 ± 0.04[b]	25.2 ± 0.19[a]	25.1 ± 0.15[a]	3.7 ± 0.41[b]
20:5n-3	n.d.[a]	n.d.[a]	1.7 ± 0.12[b]	n.d.[a]	n.d.[a]	2.9 ± 0.13[b]
22:0	0.2 ± 0.03[a]	0.2 ± 0.03[a]	0.1 ± 0.00[b]	0.3 ± 0.02	0.3 ± 0.07	0.3 ± 0.02
22:4n-6	1.7 ± 0.13[a]	2.3 ± 0.17[b]	0.2 ± 0.04[c]	2.7 ± 0.09[a]	3.0 ± 0.62[a]	0.4 ± 0.16
22:5n-6	8.6 ± 0.20[a]	11.7 ± 0.35[b]	n.d.[c]	10.1 ± 0.33[a]	13.2 ± 0.45[b]	n.d.[c]
22:5n-3	1.1 ± 0.06[a]	0.4 ± 0.16[b]	n.d.[c]	0.6 ± 0.02[a]	0.1 ± 0.07[b]	n.d.[b]
22:6n-3	8.5 ± 0.24[a]	6.5 ± 0.38[b]	44.6 ± 0.68[c]	5.2 ± 0.18[a]	3.3 ± 0.16[a]	42.0 ± 1.17[b]
24:0	0.3 ± 0.03	0.3 ± 0.05	0.2 ± 0.02	0.3 ± 0.02	0.2 ± 0.04	0.3 ± 0.02
24:1	0.2 ± 0.03	0.2 ± 0.04	0.1 ± 0.00	0.1 ± 0.02[a]	0.2 ± 0.02[b]	0.1 ± 0.02[a]
Saturates	37.5 ± 1.0	36.7 ± 1.9	36.9 ± 0.8	37.7 ± 0.6	37.5 ± 0.5	38.7 ± 1.0
MUFA	8.3 ± 0.2[a]	6.1 ± 0.2[b]	6.1 ± 0.2[b]	5.8 ± 0.2[a]	3.8 ± 0.1[b]	3.8 ± 0.2[b]
PUFA	54.5 ± 0.8	57.3 ± 0.7	57.2 ± 1.0	56.2 ± 0.7	57.3 ± 0.8	57.7 ± 1.9
n-6	44.8 ± 0.5[a]	50.3 ± 0.4[b]	10.7 ± 0.2[c]	50.4 ± 0.5[a]	53.9 ± 0.7[b]	12.6 ± 0.6[c]
n-3	9.7 ± 0.3[a]	7.0 ± 0.3[b]	46.5 ± 0.8[c]	5.8 ± 0.2[a]	3.4 ± 0.1[b]	45.1 ± 1.3[c]
n-6/n-3	4.6 ± 0.2[a]	7.2 ± 0.4[b]	0.2 ± 0.0[c]	8.7 ± 0.3[a]	15.9 ± 0.5[b]	0.3 ± 0.0[c]

[a]See Table 3 footnote.

corporation of long-chain PUFA reflective of the dietary oil used (Table 4). Oleic acid (OA, 18:0), LA, and docosatetraenoic acid (DTA) levels were modestly decreased, while AA and DPA levels were profoundly depressed (by nearly 95% for AA and to undetectable levels for DPA) in MF–DHA relative to the other groups. These decreases in n-6 PUFA are completely compensated for by substantial increases in EPA, and more importantly DHA, in MF–DHA-fed animals. DHA accounted for a remarkable 42 and 44.6% of the fatty acids in phospholipid from heart and quadriceps muscle, respectively. For muscle, this corresponds exactly to the composition of the oil supplement.

Liver fatty acid composition was also markedly affected by the dietary oil used (Table 5). OA levels were decreased in MF–DHA animals, and AA and DPA levels were markedly decreased relative to the LF–CO and MF–SO groups. Unlike the other tissues, however, LA and DTA levels were not lower in MF–DHA relative to the LF–CO and MF–SO groups. Predictably, DHA levels were much higher in the MF–DHA group compared to both n-6 rich diets. EPA levels in the liver were much higher than in any other tissue examined, suggesting that considerable retroconversion of DHA to EPA had occurred in this tissue. We also noted increases in the short-chain saturated fatty acids, (14:0) and palmitic acid (16:0) which may have been an attempt by the liver to moderate fluidity changes induced by the substantial incorporation of long-chain PUFA in response to DHA feeding.

TABLE 5
Fatty Acid Analysis of Tissues—Liver[a]

	Corn oil (n = 6)	Safflower oil (n = 6)	DHASCO (n = 6)
14:0	0.1 ± 0.02[a]	0.1 ± 0.00[a]	0.3 ± 0.02[b]
14:1	n.d.	n.d.	n.d.
16:0	17.2 ± 0.33[a]	16.4 ± 0.17[a]	23.9 ± 0.42[b]
16:1	0.2 ± 0.08[a]	n.d.	0.4 ± 0.06[c]
18:0	24.6 ± 0.40[a]	25.5 ± 0.24[a]	20.1 ± 0.35[b]
18:1	4.7 ± 0.12[a]	2.7 ± 0.21[b]	5.2 ± 0.23[a]
18:2n-6	7.1 ± 0.38[a]	8.2 ± 0.24[b]	8.4 ± 0.21[b]
18:3n-6	0.1 ± 0.03[a]	0.1 ± 0.02[a]	n.d.[b]
20:1	0.3 ± 0.02[a]	0.2 ± 0.02[b]	0.2 ± 0.02[b]
20:2n-6	0.2 ± 0.02[a]	0.5 ± 0.04[b]	0.1 ± 0.00[c]
20:3n-6	n.d.[a]	n.d.[a]	0.3 ± 0.11[b]
20:4n-6	31.3 ± 0.40[a]	31.6 ± 0.37[a]	2.1 ± 0.14[b]
20:5n-3	n.d.[a]	0.1 ± 0.02[a]	11.0 ± 0.34[b]
22:0	0.3 ± 0.00[a]	0.3 ± 0.02[a]	0.2 ± 0.00[b]
22:4n-6	n.d.[a]	0.9 ± 0.29[b]	n.d.[a]
22:5n-6	6.7 ± 0.12[a]	8.5 ± 0.56[b]	0.3 ± 0.04[c]
22:5n-3	n.d.	n.d.	n.d.
22:6n-3	5.0 ± 0.20[a]	3.1 ± 0.09[b]	26.5 ± 0.38[c]
24:0	1.1 ± 0.04[a]	1.1 ± 0.05[a]	0.9 ± 0.04[b]
24:1	0.4 ± 0.03[a]	0.4 ± 0.02[a]	0.3 ± 0.02[b]
Saturates	43.5 ± 0.8	43.6 ± 0.5	45.6 ± 0.8
MUFA	5.6 ± 0.2[a]	3.3 ± 0.2[b]	6.1 ± 0.3[a]
PUFA	50.6 ± 0.4[a]	53.2 ± 0.3[b]	49.0 ± 0.7[a]
n-6	45.4 ± 0.3[a]	49.8 ± 0.2[b]	11.2 ± 0.2[c]
n-3	5.2 ± 0.1[a]	3.4 ± 0.1[b]	37.8 ± 0.5[c]
n-6/n-3	8.5 ± 0.2[a]	15.1 ± 0.4[b]	0.3 ± 0.0[c]

[a]See Table 3 footnote.

TABLE 6
Fatty Acid Analysis of Tissues—Red Blood Cells and Bone Marrow[a]

	Red blood cells			Bone marrow		
	Corn oil (n = 6)	Safflower oil (n = 6)	DHASCO (n = 6)	Corn oil (n = 6)	Safflower oil (n = 6)	DHASCO (n = 6)
14:0	0.2 ± 0.03[a]	0.2 ± 0.07[a]	0.6 ± 0.14[b]	0.7 ± 0.10[a]	0.7 ± 0.14[a]	1.5 ± 0.06[b]
14:1	0.1 ± 0.05	n.d.	0.1 ± 0.02	0.3 ± 0.07	0.4 ± 0.09	0.3 ± 0.09
16:0	24.8 ± 0.53[a]	23.7 ± 0.52[a]	29.2 ± 0.27[b]	24.1 ± 1.12	25.8 ± 1.72	26.2 ± 0.64
16:1	0.2 ± 0.04	0.1 ± 0.02	0.2 ± 0.03	0.8 ± 0.29[a]	n.d.[b]	0.8 ± 0.19[a]
18:0	18.9 ± 0.83[a]	18.8 ± 0.44[a]	13.3 ± 0.42[b]	17.4 ± 0.45[a]	17.8 ± 0.27[a]	15.0 ± 0.58[b]
18:1	7.9 ± 0.25[a]	6.2 ± 0.14[b]	8.9 ± 0.13[c]	12.5 ± 0.27[a]	9.3 ± 0.37[b]	14.2 ± 0.25[c]
18:2n-6	6.5 ± 0.17[a]	8.6 ± 0.08[b]	8.3 ± 0.17[b]	7.7 ± 0.37[a]	10.2 ± 0.74[b]	9.9 ± 0.43[b]
18:3n-6	0.1 ± 0.02[a]	0.1 ± 0.02[a]	n.d.[b]	0.1 ± 0.02[a]	0.1 ± 0.02[a]	n.d.[b]
20:1	0.1 ± 0.02	0.2 ± 0.04	0.2 ± 0.02	0.4 ± 0.03	0.4 ± 0.03	0.3 ± 0.03
20:2n-6	0.2 ± 0.00[a]	0.5 ± 0.02[b]	0.1 ± 0.00[c]	0.6 ± 0.03[a]	1.1 ± 0.09[b]	0.3 ± 0.00[c]
20:3n-6	0.2 ± 0.03[a]	0.1 ± 0.02[b]	0.4 ± 0.00[c]	0.4 ± 0.05[a]	0.3 ± 0.02[a]	0.9 ± 0.05[b]
20:4n-6	23.7 ± 0.38[a]	22.8 ± 0.25[b]	3.2 ± 0.16[c]	23.7 ± 0.56[a]	21.6 ± 1.32[a]	4.1 ± 0.40[b]
20:5n-3	n.d.[a]	n.d.[a]	7.1 ± 0.04[b]	0.1 ± 0.02[a]	0.2 ± 0.11[a]	6.3 ± 0.59[b]
22:0	0.5 ± 0.04	0.6 ± 0.05	0.5 ± 0.05	0.5 ± 0.02	0.5 ± 0.04	0.5 ± 0.03
22:4n-6	3.0 ± 0.16[a]	3.8 ± 0.15[b]	0.2 ± 0.02[c]	4.6 ± 0.19[a]	4.7 ± 0.29[a]	0.3 ± 0.05[b]
22:5n-6	3.0 ± 0.12[a]	3.4 ± 0.16[b]	n.d.[c]	1.9 ± 0.11[a]	2.4 ± 0.37[b]	n.d.[b]
22:5n-3	0.4 ± 0.03	0.2 ± 0.02	0.6 ± 0.19	0.3 ± 0.03[a]	0.3 ± 0.06[a]	1.1 ± 0.23[b]
22:6n-3	1.7 ± 0.42[a]	1.1 ± 0.11[a]	16.7 ± 0.35[b]	0.7 ± 0.07[a]	1.1 ± 0.37[a]	15.7 ± 1.16[b]
24:0	1.6 ± 0.12[a]	1.9 ± 0.18[a]	2.8 ± 0.31[b]	1.5 ± 0.17	1.0 ± 0.12	1.2 ± 0.13
24:1	1.0 ± 0.13	1.0 ± 0.14	1.1 ± 0.15	n.d.[a]	1.3 ± 0.10[b]	1.1 ± 0.09[b]
Saturates	46.4 ± 1.6	45.5 ± 1.3	46.8 ± 1.2	44.8 ± 1.9	46.5 ± 0.2	44.9 ± 1.5
MUFA	9.4 ± 0.5[a]	7.5 ± 0.3[b]	10.5 ± 0.4[a]	14.1 ± 0.7[a]	11.5 ± 0.6[b]	16.7 ± 0.6[c]
PUFA	37.9 ± 0.4[a]	40.8 ± 0.4[b]	36.7 ± 0.2[c]	40.4 ± 1.1	42.3 ± 2.8	40.1 ± 2.4
n-6	36.7 ± 0.3[a]	39.3 ± 0.3[b]	12.2 ± 0.2[c]	39.1 ± 1.0[a]	40.5 ± 2.3[a]	15.5 ± 1.0[b]
n-3	2.2 ± 0.1[a]	1.5 ± 0.1[b]	24.5 ± 0.2[c]	1.3 ± 0.1[a]	1.8 ± 0.5[a]	24.6 ± 1.4[b]
n-6/n-3	16.7 ± 0.4[a]	29.4 ± 2.4[b]	0.5 ± 0.0[c]	30.1 ± 3.1[a]	34.9 ± 8.0[a]	0.6 ± 0.1[b]

[a]See Table 3 footnote.

The final two tissues we examined were red blood cells and bone marrow (Table 6). Both tissues, like the others described, were responsive to dietary lipids. We observed lower concentrations of stearate, and markedly lower AA, DTA, and DPA levels in the MF–DHA relative to both the LF–CO and MF–SO groups, with little difference between blood and bone marrow. DHA and EPA levels were also much higher in DHA-fed animals relative to the other n-6 rich groups.

Overall, the fatty acid profiles of the various tissues appear to be substantially altered by dietary fatty acids. Although differences in fatty acid compositions between the LF–CO and MF-SO group were observed, these were often very small, particularly when compared to the MF–DHA group. No differences in fatty acid composition were observed for animals treated with the chemotherapeutic agent, araC (not shown). Thus, the major determinant of the lipid composition of the various tissues appeared to be the quality of the fat in the diet.

Because one of the long-term goals of this research is to examine the utility of lipid supplementation both as an adjuvant in cancer chemotherapy and also potentially as a chemopreventive agent, it was important to know what the effects of DHA might be on some host tissues. Since one of the major deleterious consequences of drug therapy is bone marrow suppression, we examined not only the fatty acid composition but also the bone marrow cellularity, colony-forming ability, and the Coulter channelizer profiles of the extracted bone marrow.

All animals treated with araC exhibited moderate neutropenia with no differences between diet groups (not shown). However this observation alone tells us little about the ability of this animal to repopulate this compartment. A more direct measure of this capacity is assessment of the bone marrow compartment. Total bone marrow yield was substantially affected by the level of fat in the diet. The untreated, MF–DHA group had twice the number of bone marrow cells per femur, whether expressed as cells/femur or cells/femur/g body weight when compared to chow-fed animals or those on the LF–CO diet (Fig. 1A). There was also a trend for the DHA-fed animals to have a higher bone marrow cellularity than the MF–SO group (P = 0.068). Although the differences in the araC-treated groups were not statistically significant, once again there was a trend for the MF–DHA-fed animals to have higher bone marrow yields than both the n-6 rich diets (P = 0.077 vs. MF–SO and P = 0.10 vs. LF–CO). Both MF groups treated with araC had lower bone marrow cell yields than their saline-injected counterparts, and although the araC LF–CO group appeared to have bone marrow cell numbers that were lower than saline-injected animals, this did not reach statistical significance (Fig. 1A).

We measured the ability of these bone marrow preparations to support the formation of CFU–GM. Aliquots of

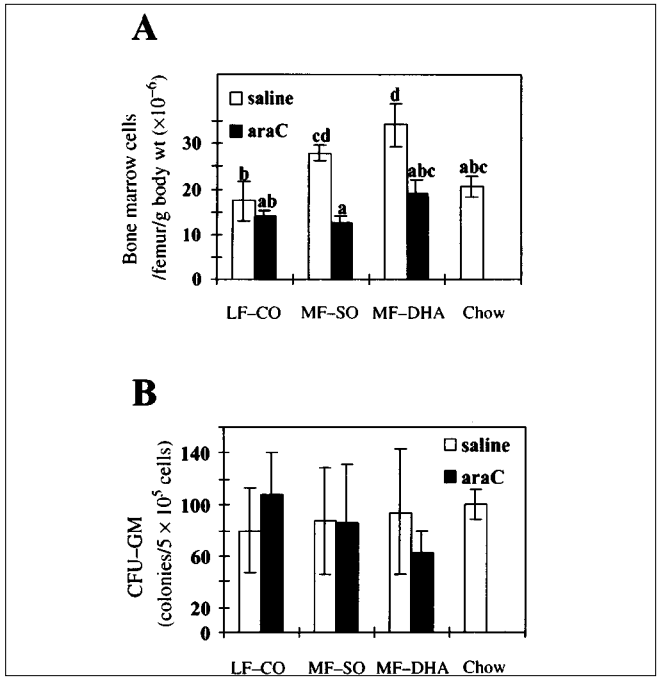

FIG. 1. Bone marrow cellularity and granulocyte/macrophage colony-forming ability. Seven days before the end of the trial animals were injected daily with saline or 50 mg/kg arabinosylcytosine as described in the Materials and Methods section. Injections were stopped 24 h before animals were sacrificed. On the final experimental day, bone marrow was harvested and the total number of cells per femur estimated by Coulter counting (A). Following washing, three aliquots of each bone marrow sample were plated in methylcellulose and granulocyte/macrophage colonies counted seven days later (B). Bars represent the mean values ± standard error of the mean. Bars not sharing a letter are statistically different by analysis of variance followed by Student Newman-Keuls post-test, $P < 0.05$. Low fat–corn oil (LF–CO)-saline, n = 8; LF–CO-arabinosylcytosine (araC), n = 9; moderate fat–safflower oil (MF-SO)-saline, n = 9; MF–SO-araC, n = 9; MF-docosahexaenoic acid (DHA)-saline, n = 9; MF–DHA–araC, n = 9; chow, n = 11; CFU–GM, colony-forming units–grandulocyte macrophage.

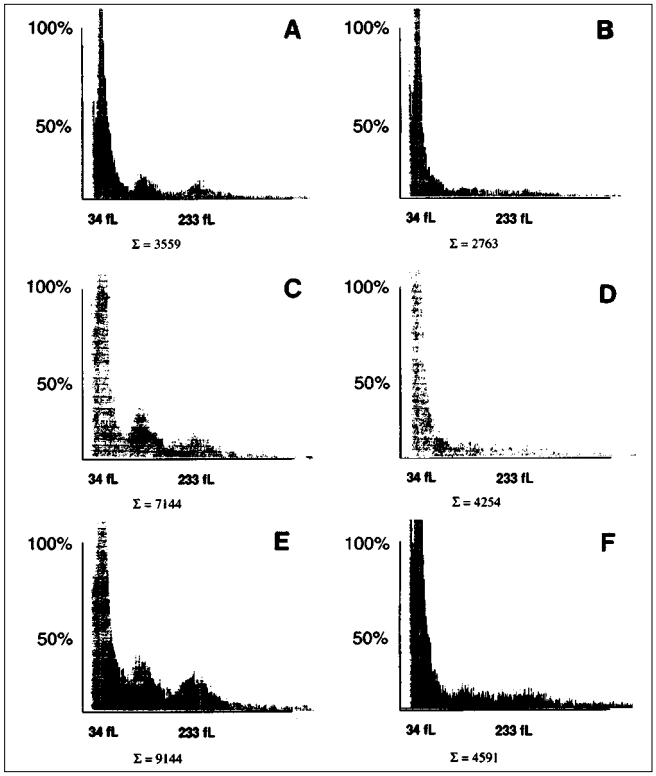

FIG. 2. Coulter channelizer distributions for bone marrow cell populations. Bone marrow was recovered from the femur of each animal as described in the Materials and Methods section. After producing a single cell suspension, cells were counted on a Coulter counter (model ZM) equipped with a 256-channel analyzer. Shown are distributions for representative, nontumor-bearing animals, with the percentage of the population on the y-axis and the approximate cell volume on the x-axis. The two cursor lines mark volumes of 34 and 233 fL, for the left and right cursor, respectively. The total number of events recorded is shown by the Σ =. (A) LF–CO; (B) LF–CO–araC; (C) MF–SO; (D) MF–SO–araC; (E) MF–DHA; (F) MF–DHA–araC.

washed bone marrow cells were plated in methylcellulose and colonies counted a week later. No differences in the colony-forming ability were observed for any of the dietary groups, nor did a single course of araC treatment appear to target the granulocyte/macrophage precursor population specifically (Fig. 1B). Thus, the differences in total bone marrow cellularity could not be explained by differences in the relative proportion of CFU–GM.

Qualitatively the Coulter channelizer profiles from the different dietary groups showed that araC-treated animals had lost populations of larger cells to differing degrees depending on the dietary treatment (Fig. 2). DHA-fed animals appeared to retain more of these larger cells following araC treatment than did the other two groups. The identity of these populations remains undetermined but, as demonstrated above, these would not seem to represent GM precursors, as we found no differences in colony-forming ability between the different diet and drug treatment groups.

DISCUSSION

Feeding DHASCO™ at 10% by weight (25% of calories) results in a 50% decrease in total cholesterol compared to a LF diet and a 40% decrease compared to a LA-rich diet. Diets rich in either LA or DHA reduce serum triglycerides by 50% compared to a LF diet. The hypotriglyceridemic effect is one that is common to both human trials feeding fish/fish oil and trials feeding fish oils, and now DHA, to rats (4). Although we can not exclude the involvement of EPA in this process (because of substantial retroconversion of DHA to EPA), the fact that a supplement containing no EPA itself could achieve this means that EPA need not be present in the diet to meet these objectives. Our results are similar to those reported by Ikeda and coworkers (22) who showed that adding DHA (as the ethyl ester) at 1% of the diet reduced triglyceride levels to ~118 mg/dL and cholesterol to 5.7 mg/dL in growing Sprague-Dawley rats. However, our results contrast those of Willumsen and coworkers (23), who recently suggested that purified DHA (92%) had no triglyceride-lowering activity.

The major difference between our study and theirs is that they used purified DHA as a supplement in a soya oil-based diet while we used a triglyceride preparation of DHA as the major oil in our diet. The DHASCO™, in addition to containing DHA, also contains a substantial portion of saturated fatty acids (42%) which could have interacted with the DHA to produce the observed hypocholesterolemic and hypotriglyceridemic effects observed in our study. This is supported by a study by Garg *et al.* (24), showing that fish oil dramatically lowered serum triglyceride levels in rats fed a high saturated fatty acid diet. The interaction between the saturated fatty acids and DHA needs to be further studied.

We were able to profoundly modify the fatty acid compositions of a wide variety of tissues with DHA feeding at relatively modest levels of total dietary fat (10% by weight). In fact, the changes we have observed in this *in vivo* model are even larger than we have observed in *in vitro* cell culture models, where DHA incorporation is at best 18% of total phospholipid (18). Therefore substantive changes in lipid composition are clearly achievable *via* the dietary route of administration. Considerable intertissue variation was observed, particularly in the n-6 rich diets. The high variability between tissues in the n-6/n-3 ratio observed in either the LF–CO or MF–SO diets was normalized by DHASCO™ feeding. Thus, tissues appear to be unable to resist n-3 substitution for n-6 when presented with high circulating n-3 fatty acids.

The fatty acid composition of skeletal and cardiac muscle was the most sensitive to changes in dietary fatty acids and contains as much as 46% DHA in membrane phospholipids. We have recently shown that basal- and hormone-stimulated glucose transport is altered in the adipocytes of MF–DHA-fed animals (25) and have shown that *in vitro* supplementation of DHA results in increased transport of purine nucleosides in leukemic cells (26). We also recently showed that nucleoside transport in red blood cells from humans given fish oil supplements is lower than in the unsupplemented condition and that only modest changes in the red cell fatty acid composition were necessary to achieve this (16). However, it remains to be seen whether the profound fatty acid changes we have induced here translate into altered muscle cell function or altered function of any of the other tissues besides adipose. The observation that nucleoside transport can be modified by n-3 feeding is relevant to the current study in that araC (used here) is a commonly used chemotherapeutic drug and therefore factors that affect its transport/metabolism will impact on both tumor and normal cell toxicity.

Our study shows that bone marrow readily incorporates DHA and in skeletal and cardiac muscle to even higher levels. The DHASCO™ contains 42.5% saturated fatty acids, but the content of saturated fatty acids in most of the tissues (i.e., the bone marrow) of the MF–DHA animals was not different from the LF–CO or MF–SO groups. Although Burns and Spector (27) have proposed that bone marrow is more disposed to incorporate saturated fatty acids into phospholipid, this conclusion may have been based on studies comparing incorporation in coconut oil and sunflower oil fed animals (28). Since the long-chain PUFA and saturates were not presented simultaneously in these studies, this could have been why bone marrow appeared to prefer saturated fatty acids when compared to other tissues. Our study directly compared levels of incorporation of n-3 and saturated fatty acids. Therefore, one would have to conclude that given a choice, many tissues will preferentially incorporate n-3 PUFA over saturated fatty acids into membrane phospholipid.

One of the major novel findings of this study was the remarkable effect of the level of fat in the diet on bone marrow cellularity. We are not aware of previous reports documenting an effect of lipid level or fat type on bone marrow development in the post-weanling rat. A twofold increase in the number of bone marrow cells, without a change in the proportion of GM precursors means that the MF–DHA and MF–SO animals have nearly twice as many GM precursors per animal (or per g body weight). It is likely that this would have consequences on immune function and disease resistance as well as the capacity to maintain normal circulating blood cell populations. In the current study only a single time point (at necropsy) was examined for peripheral neutrophil counts demonstrating that all araC animals were neutropenic to the same extent. In future studies, it will be important to look at how quickly the animals recover from aggressive chemotherapy. We also observed differences between dietary groups, on the level of bone marrow suppression seen following chemotherapeutic challenge. Bone marrow from LF–CO animals appeared to be less sensitive to the S-phase specific drug, araC, when compared to both moderate fat groups. We believe this to be an actual loss of bone marrow cells rather than a failure of the moderate fat diets to allow bone marrow expansion in the presence of araC. We conclude this based on the fact that the araC treatment period is only six days and unlikely to be sufficient time for the level of bone marrow expansion required to achieve these differences in final cell number. Having established a model which results in substantial changes in tissue fatty acid incorporation, we can now examine the effect this has on the sensitivity of these normal tissues and a transplantable tumor to aggressive chemotherapeutic drug treatment. Our first trial suggests that DHASCO™ feeding increases the number of bone marrow cells and specifically the number of CFU–GM. Our early araC results also suggest that a larger proportion of bone marrow precursor cells are killed in the moderate fat dietary groups compared to the LF group. Despite the large loss in bone marrow cells in DHASCO™-fed animals, the compartment remaining appears to be indistinguishable from the LF araC-treated group and from untreated chow-fed animals. Future experiments will address whether increased CFU–GM is a regular pattern during DHASCO™ feeding, whether differences in drug sensitivity between diet groups will be maintained at a higher araC doses and whether the DHA/EPA content established in skeletal muscle following DHASCO™ feeding has impact on muscle metabolism/function.

We observed no overt signs of toxicity of DHA in any of the animals over a relatively long time period (8–9 wk), nor

did we observe any growth inhibition or change in the behavior of the animals. However, we have previously reported that the MF–DHA diet does produce glucose intolerance in these animals and reduced hormonal stimulation of glucose transport in adipocytes (25). Therefore, not all of the effects of DHA are positive in terms of health. However, one should keep in mind that the levels used here are 10–100 times the levels previously included in animal diets for toxicity testing of the DHASCO™ supplement (29). The goal of this research is to investigate the potential role for DHA-containing oils as adjuvants for chemotherapy. The levels being used here are clearly in the therapeutic range, and therefore the risk/benefit is a more relevant measure of efficacy.

In conclusion, DHA induces profound hypotriglyceridemia and hypocholesterolemia in post-weanling Fischer 344 rats compared to a LF n-6 diet. In addition to its lipid-lowering activity, we also demonstrated that DHA feeding has substantial bone marrow enhancing activity, resulting in a twofold increase in cell numbers over n-6 or chow-fed animals. Further examination of DHASCO™ as a supplement to animal and possibly human diets, or in pharmacologic doses as part of a chemotherapy regime for cancer treatment is certainly warranted.

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Oryzanol Decreases Cholesterol Absorption and Aortic Fatty Streaks in Hamsters

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ABSTRACT: Oryzanol is a class of nonsaponifiable lipids of rice bran oil (RBO). More specifically, oryzanol is a group of ferulic acid esters of triterpene alcohol and plant sterols. In experiment 1, the mechanisms of the cholesterol-lowering action of oryzanol were investigated in 32 hamsters made hypercholesterolemic by feeding chow-based diets containing 5% coconut oil and 0.1% cholesterol with or without 1% oryzanol for 7 wk. Relative to the control animals, oryzanol treatment resulted in a significant reduction in plasma total cholesterol (TC) (28%, $P < 0.01$) and the sum of IDL-C, LDL-C, and VLDL-C (NON-HDL-C) (34%, $P < 0.01$). In addition, the oryzanol-treated animals also exhibited a 25% reduction in percent cholesterol absorption vs. control animals. Endogenous cholesterol synthesis, as measured by the liver and intestinal HMG-CoA reductase activities, showed no difference between the two groups. To determine whether a lower dose of oryzanol was also efficacious and to measure aortic fatty streaks, 19 hamsters in experiment 2 were divided into two groups and fed for 10 wk chow-based diets containing 0.05% cholesterol and 10% coconut oil (w/w) (control) and the control diet plus 0.5% oryzanol (oryzanol). Relative to the control, oryzanol-treated hamsters had reduced plasma TC (44%, $P < 0.001$), NON-HDL-C (57%, $P < 0.01$), and triglyceride (TG) (46%, $P < 0.05$) concentrations. Despite a 12% decrease in high density lipoprotein cholesterol (HDL-C) ($P < 0.01$), the oryzanol-treated animals maintained a more optimum NON-HDL-C/HDL-C profile (1.1 ± 0.4) than the control (2.5 ± 1.4; $P < 0.0075$). Aortic fatty streak formation, so defined by the degree of accumulation of Oil Red O-stained macrophage-derived foam cells, was reduced 67% ($P < 0.01$) in the oryzanol-treated animals. From these studies, it is concluded that a constituent of the nonsaponifiable lipids of RBO, oryzanol, is at least partially responsible for the cholesterol-lowering action of RBO. In addition, the cholesterol-lowering action of oryzanol was associated with significant reductions in aortic fatty streak formation.

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Abbreviations: HDL-C, high density lipoprotein cholesterol; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase; IDL-C, intermediate density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; NON-HDL-C, the sum of IDL-C, LDL-C, and VLDL-C; PBS, phosphate-buffered saline; RBO, rice bran oil; TC, total cholesterol; TG, triglyceride; TLC, thin-layer chromatography; VLDL-C, very low density lipoprotein cholesterol.

The cholesterol-lowering effect of rice bran oil (RBO) has recently been reported by several investigators (1–7). A number of studies have suggested that the nonsaponifiables in the RBO may play a very important role in the cholesterol-lowering effect of RBO (1–3,5–8). When the nonsaponifiable lipids extracted from RBO were fed to rats in an amount equivalent to that in the RBO (2), similar cholesterol-lowering effects were observed. When oryzanol, a constituent of the nonsaponifiable lipids in RBO, was added to a diet containing purified RBO (8), it further decreased the serum total cholesterol (TC) level in rats compared to purified alone, suggesting that the hypocholesterolemic activity of RBO is attributable to oryzanol and to some other components in the nonsaponifiable matter. A study in nonhuman primates demonstrated that serum cholesterol levels decreased as the level of RBO in the diet increased, and this reduction in serum cholesterol level was not entirely explained by the fatty acid composition of the dietary lipids, suggesting a component of RBO other than its fatty acid content was responsible for its hypocholesterolemic effect (5).

RBO, unlike most vegetable oils, contains a relatively high proportion of nonfatty acid components (the nonsaponifiable lipids). Refined RBO may contain 1.5–2.6% of nonsaponifiable lipids, and crude RBO may contain up to 5% of nonsaponifiable lipids (9). In contrast, most common refined vegetable oils contain only 0.3–0.9% of nonsaponifiable lipids (10). The nonsaponifiables of RBO contain sterols (43%), triterpene alcohols (28%), 4-methyl sterols (10%), and less polar components (19%) (11). In the crude oil, about 20% of the nonsaponifiables of RBO are in the form of ferulic acid esters, which are a mixture of esters of ferulic acid (4-hydroxy-3-methoxycinnamic acid) with sterols (16% campesterol, 7% β-sitosterol) and triterpene alcohols (30% cycloartenol, 23% 24-methylene-cycloartanol, and 22% cyclobranol) (11). This mixture of ferulic acid esters has been named oryzanol since it was originally found in RBO and contained a hydroxyl group. The structure of oryzanol is shown in Figure 1. Recently, it has been reported that oryzanol also has a cholesterol-lowering property when fed to humans (12–14) and animals (15–17).

A recent study in our laboratory demonstrated that physically refined RBO, which can contain up to 3.5% nonsaponifi-

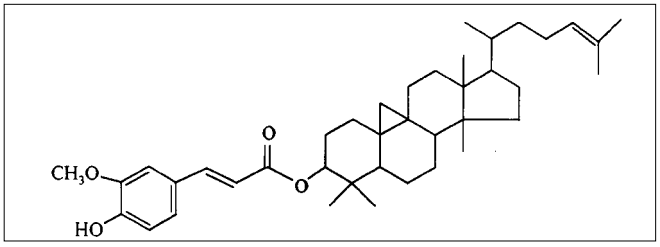

FIG. 1. Oryzanol comprises any of several plant sterols esterified to ferulic acid; the example shown here is cycloartenyl ferulate.

able components and more than 10 times the oryzanol content than alkali-refined RBO, prevented diet-induced hypercholesterolemia by inhibiting dietary cholesterol absorption and enhancing fecal neutral sterol excretion (7). These types of changes were associated with reductions in early atherosclerosis.

The present studies were designed to examine the effect of oryzanol, one of the nonsaponifiable components in RBO, on whole body cholesterol metabolism and early atherosclerosis by measuring: (i) plasma lipid and lipoprotein levels; (ii) dietary cholesterol absorption; (iii) liver and intestinal cholesterol synthesis; and (iv) aortic fatty streak formation as defined by accumulation of Oil-Red-O positive macrophage-derived foam cells. The results of our studies suggest that oryzanol feeding prevented the typical plasma TC and the sum of intermediate density lipoprotein cholesterol (IDL-C), low density lipoprotein cholesterol (LDL-C), and very low density lipoprotein cholesterol (VLDL-C) (NON-HDL-C) increase and aortic fatty streak formation associated with dietary saturated fat and cholesterol feeding. These effects may be partially explained by the ability of oryzanol to inhibit cholesterol absorption.

MATERIALS AND METHODS

Experiment 1. Animals. Thirty-two male F_1B Golden hamsters, 8 wk of age (Biobreeder, Fitchburg, MA), were used in this experiment. The study was approved by the Institutional Animal Care and Use Committees at The University of Massachusetts-Lowell and at the USDA Human Nutrition Research Center on Aging at Tufts University. Animals were individually housed in hanging cages in a 12/12 light/dark cycle with the light cycle starting at 1500 h.

Experimental protocol and diets. After arrival, animals were acclimatized for 3 d with regular Purina Rodent Chow. They were then fed a hypercholesterolemic diet (control) containing 0.1% cholesterol and 5% coconut oil (Pureco 76, Capital City) added to the Purina Rodent Chow (Purina 5001; Purina Mills Inc., St. Louis, MO) for 2.5 wk to acclimatize the animals to diet. Baseline values of plasma TC, high density lipoprotein cholesterol (HDL-C), and triglycerides (TG) were measured at 2.5 wk. The NON-HDL-C was calculated as TC minus HDL-C. This plasma NON-HDL-C value was used to divide animals into two groups of of 16 with similar starting NON-HDL-C values, each fed the control or the oryzanol diet for the next 8.5 wk. The oryzanol group was fed the same control diet but with 1.0% oryzanol added at the expense of Chow. Both cholesterol and oryzanol (TSUNO, Osaka, Japan) were dissolved in coconut oil heated to 95°C. The oil mixture was mixed with powdered Chow soon after the cholesterol and oryzanol were dissolved in the oil. A chow-based, rather than a semipurified diet, was used because published data from our laboratory (18) and those from another (19) have demonstrated that the chow-based diet produced a lipoprotein profile (predominately NON-HDL-C) more similar to humans. All diets and water were fed *ad libitum* throughout the study except as otherwise noted. Blood samples were drawn at 5 and 7 wk of the treatment period for plasma lipid and lipoprotein determination. At week 8, cholesterol absorption was measured, after which, animals were euthanized and their livers and small intestines collected for measurement of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase activity.

Chemicals. Cholesterol and TC reagents were purchased from Abbott (Chicago, IL). HDL-C precipitating reagent ($PTA/MgCl_2$) was from Sigma Chemical (St. Louis, MO). Calibration standards for cholesterol, HDL-C, and TG were from Verichem Labs (Providence, RI). Controls for cholesterol, HDL-C, and TG were from Soloman Park Labs (Kerkland, WA). [4-^{14}C]-Cholesterol (57.1 mCi/mmol) and [1,2-^{3}H(N)]-cholesterol (51.3 Ci/mmol) were from New England Nuclear (Boston, MA). Carrier cholesterol was from Sigma Chemical Co. Absolute ethanol (200 proof) was from Aaper Alcohol and Chemical Co (Shelbyville, KY), and Ensure® liquid diet was from Ross Laboratory (Columbus, OH). Glucose-6-phosphate, $NADP^+$, glucose-6-phosphate dehydrogenase, trypsin inhibitor (soybean trypsin inhibitor, type 1-s) were from Sigma Chemical Co. [3-^{14}C] HMG-CoA (57 mCi/mmol], [5-^{3}H] mevalonolactone (24 Ci/mmol), and Aquasol-2 were from New England Nuclear (Boston, MA). Silica gel thin-layer chromatography (TLC) plates were from Eastman Kodak (Rochester, NY). Fetal bovine serum protein standard and Coomassie Blue dye reagent were from Bio-Rad Laboratories (Richmond, CA). Oryzanol was from TSUNO Inc.

Plasma lipid and lipoprotein determination. After an overnight fast, hamsters were tranquilized with 50% CO_2/ 50% O_2 and blood samples drawn from the retro-orbital sinus into Eppendorf tubes containing 5 µL heparin. After centrifugation at 4000 rpm for 8 min, plasma was collected for TC, NON-HDL-C, HDL-C, and TG measurements. TC (20) and TG (21) were quantified using enzymatic methods, and HDL-C was estimated after phosphotungstate precipitation (22) of VLDL-C and LDL-C. The NON-HDL-C fraction was determined by the difference between TC and HDL-C and includes IDL-C, VLDL-C, and LDL-C. The assays were standardized by participation in the Centers for Disease Control—National Heart, Lung and Blood Institute Standardization Program.

Measurement of cholesterol absorption. (i) Preparation of oral dose (per hamster): The percentage of an oral dose of cholesterol absorbed was measured by the dual-isotope plasma ratio method described by Zilversmit *et al*. (23,24), with minor modifications. [4-^{14}C] Cholesterol (0.5 μCi) containing 5.0 mg cholesterol was dissolved in 400 μL absolute ethanol. The ethanol mixture was evaporated to dryness under a stream of nitrogen. For the control dose, the content of the dry tube was redissolved in 250 μL corn oil. For the experimental dose, 50 mg oryzanol was dissolved in the 250 μL corn oil. The oil mixtures were then suspended with an aliquot of liquid diet (2 mL Ensure®) by sonication (Polytron, Brinkmann Instruments Inc., Westbury, NY) for 2 min. The amount of fat, cholesterol, and oryzanol included with the tracer dose mimicked that in the diet in a 24-h period, had the animals been allowed to eat. The oral doses were prepared a few hours before use. (ii) Preparation of intravenous (IV) dose (per hamster): [1,2-^{3}H] Cholesterol (1.0 μCi) containing 6.5 μg cholesterol was dissolved in 30 μL absolute ethanol. Colloidal cholesterol was prepared by adding 0.37 mL sterile saline just prior to dosing. (iii) Administration of oral and IV dose: All animals were fasted 18 h (starting at the beginning of the dark cycle) prior to oral and IV dosing with the isotopes. Hamsters were anesthetized with 50% CO_2/50% O_2. The external jugular vein of the hamster was exposed under sterile conditions and the IV isotope dose (approximately 0.4 mL) was injected into the hamster. The oral isotope dose (about 2 mL) was gavaged to the stomach immediately following the injection of the IV dose. Food was withheld for an additional 6 h before animals were fed their experimental diets. Blood samples were drawn from the retro-orbital sinus 72 h after dosage. Preliminary data showed a plateau by 48 h after dosing. The percentage cholesterol absorption was calculated based on the plasma isotope ratio of the oral (^{14}C) and IV (^{3}H) doses (23).

Measurement of HMG-CoA reductase activity. (i) Buffered solutions: TEDK buffer contains 50 mM Tris-HCl (pH 7.5), 1.0 mM EDTA, 5.0 mM dithiothreitol, and 70 mM KCl. TEDF buffer contains 50 mM Tris-HCl (pH 7.5), 1.0 mM EDTA, 5.0 mM dithiothreitol, and 70 mM NaF. TEDK-TI (trypsin inhibitor) buffer contains TEDK with 0.04% trypsin inhibitor (0.4 mg/mL). (ii) Preparation of hepatic microsomes: Hepatic microsomes for measurement of hepatic HMG-CoA reductase activity were prepared as described by Harwood *et al*. (25) with minor modification. Immediately after each hamster was killed in a chamber filled with 100% CO_2, a piece of liver (1.0 g) was removed and homogenized at 4°C in TEDK and TEDF buffers (3 mL buffer per gram liver), using a Dounce tissue homogenizer (15 strokes) with a loose pestle. Homogenates were first centrifuged at 10,000 × *g* (4°C, 15 min), and the resultant supernatant was centrifuged at 175,000 × *g* (4°C, 90 min). The resulting microsomal pellets were lightly rinsed and resuspended in the TEDK and TEDF buffers (3.0 mL buffer per gram liver), using a Dounce tissue homogenizer (10 strokes) with a tight pestle. The microsomes were then stored at −70°C before further analysis. The protein contents of the microsomes were determined by the method of Bradford (26), using bovine serum albumin as standard. (iii) Preparation of intestinal microsomes: Intestinal microsomes were prepared for the assay of total HMG-CoA reductase activity. Microsomes were prepared from intestinal mucosal scrapings based on the method described by Harwood *et al.* (27) with minor modification. Intestinal segments from the whole small intestine (from the pylorus to the ileojunction) were removed from animals, split open longitudinally and rinsed free of fecal material with 4°C saline. The mucosa was then scraped free from the vasculature with two glass slides on a piece of ice-cold glass. The scrapings were immediately homogenized with 4 mL TEDK-TI buffer using a Dounce homogenizer with a loose pestle. The homogenates were further processed for microsomes similar to the preparation of hepatic microsomes with the exception that the final microsomal pellets were resuspended in TEDK-TI buffer (3 mL per scraping), and stored at −70°C. The protein concentrations of intestinal microsomes were also determined by the Bradford protein assay (26). (iv) Measurement of hepatic and intestinal HMG-CoA reductase activity: HMG-CoA reductase (EC 1.1.1.34) activity was measured as described by Harwood *et al.* (27). Microsomal proteins, 75 μg (50 μL) from hepatic microsomes, and 50 μg (50 μL) from intestinal microsomes were preincubated for 30 min at 37°C. Following the preincubation, the reaction of HMG-CoA reductase was initiated by adding 25 μL cofactor cocktail containing 3.4 mM $NADP^+$, 30 mM glucose-6-phosphate, 0.2 unit glucose-6-phosphate dehydrogenase, 66.7 μM DL-3-[glutaryl-3-^{14}C]-HMG-CoA (12 cpm/pmol), 12,000–13,000 cpm RS-[5-^{3}H(N)] mevalonate (27.8 Ci/mmol) as an internal standard, and 68 mM EDTA. The microsomes were incubated for 40 min (37°C) for the measurement of both hepatic and intestinal HMG-CoA reductase activity. At the end of incubation, 10 μL of 6 N HCl was added to terminate the reaction and to bring about the cyclization of mevalonate into mevalonolactone. The reaction vials were then centrifuged (Beckman microcentrifuge, 5 min, setting 12) and the supernatant applied to silica gel TLC. After developing the TLC (toluene/acetone 1:1), the region containing mevalonolactone (R_f 0.4 ~ 0.8) was removed and counted for ^{3}H and ^{14}C. HMG-CoA reductase activity is expressed as pmoles of mevalonate formed per minute per mg microsomal protein.

Experiment 2. Animals. Nineteen 10-week-old male F_1B golden hamsters (Biobreeder, Fitchburg, MA) were used in this experiment. The study was approved by the Institutional Animal Care and Use Committee at the University of Massachusetts-Lowell. Animals were individually housed in hanging cages in a 12/12 h light/dark cycle. All diets were fed *ad libitum* throughout the study.

Diet. Animals were fed for 10 wk a chow-based diet (for reasons given in experiment 1) composed of 10% coconut oil (w/w) and 0.05% cholesterol as the control diet to which was added 0.5% oryzanol.

Plasma lipid and lipoprotein measurement. At 4, 8, and 10

wk on treatment, blood samples were withdrawn and plasma was prepared and analyzed for TC, HDL-C, NON-HDL-C, and TG as described in experiment 1.

Fixation of the aorta. Hamsters were anesthetized with an IP injection of sodium pentobarbital. The animals were cut open and their hearts were exposed. Blood was collected through a cardiac puncture in the left ventricle, and the right atrium was also punctured to provide an outlet for the fixative. A perfusion needle was inserted into the left ventricle and the fixative, 4% formaldehyde in phosphate-buffered saline (PBS), was allowed to flow through the animal's cardiovascular system with physiological pressure. After two minutes, the flow was slowed and fixation was continued for another 25 min. The heart and aorta were removed and fixation was completed by pinning down in its natural shape in fixative overnight. The specimens were stored in a vial containing PBS for subsequent analysis.

Aortic morphometric analysis. The heart and the descending aorta were removed and discarded. The aortic arch was rinsed with PBS and dropped in a vial containing Oil Red O stain (0-0625; Sigma Chemical Co.) in isopropanol for 15 min. The arch was cleaned by carefully removing all the Oil Red O-stained tissue from its surface. The arch was then cut vertically into two pieces which were opened longitudinally and mounted on a glass slide with their epithelial sides up. Specimens were covered with cover slips and observed under 100× magnification. An image analysis system (Image Technology Corp., Cresskill, NJ), which is attached to a light microscope, was used to measure the total Oil Red O-stained macrophage-derived foam cells which constituted the fatty streak in the aortic arch. Units of measurement of fatty streak area are $\mu m^2/mm^2 \times 10^3$ of aortic tissue.

Statistical analysis. Statistical Analysis System (SAS-PC) (28) was used for all statistical analysis. Data are presented as mean ± standard error of the mean. A paired *t*-test ($P < 0.05$) was used to determine the statistical significance of mean values between the two groups for all variables. To normalize the data and stabilize the variance, it was necessary to transform the plasma TC, NON-HDL-C, and TG data to their $\log_{10}$ values before the statistical analysis.

RESULTS

Experiment 1. Effects of oryzanol on plasma lipids, lipoproteins and body weight. Following 2.5 wk of basal diet, animals were sorted into the control and the oryzanol groups with similar plasma NON-HDL-C concentration (340 mg/dL) to start the experimental period. Although measurements were performed at both weeks 5 and 7, only data from the seventh week were used since the other dependent variables were measured after the seventh week. The oryzanol group showed a significant reduction in plasma TC (28%) and NON-HDL-C concentrations (34%) with respect to control ($P < 0.01$) (Table 1). There were no statistically significant differences in plasma HDL-C and TG levels although the latter were reduced 33% in the oryzanol group relative to the control. There was also no significant difference between the body weights of the control (111 ± 3) and oryzanol (113 ± 2) groups at the end of the experiment.

TABLE 1
Plasma Lipid, Lipoprotein, and Body Weight Values in Hamsters Fed Control and Oryzanol Diets in Experiment 1[a]

	Group	
	Control	Oryzanol
TC	342 ± 32[a]	245 ± 6[b]
NON-HDL-C	273 ± 32[a]	179 ± 7[b]
HDL-C	70 ± 2	66 ± 2
TG	237 ± 45	158 ± 10
Body weight (g)	111 ± 2	113 ± 1

[a]Values (mg/dL) are expressed as mean ± SEM ($n = 16$). Values not sharing a common superscript are significantly different at $P < 0.01$. Measurements were taken 7 wk after the initiation of the control and oryzanol diet groups. TC, total cholesterol; NON-HDL-C, the sum of intermediate density lipoprotein cholesterol, low density lipoprotein cholesterol, and very low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglyceride.

Effects of oryzanol on cholesterol absorption. Cholesterol absorption following 8 wk of dietary treatment with oryzanol was significantly reduced (25%) compared to the control group (Table 2).

Effects of oryzanol on hepatic and intestinal cholesterol synthesis. Although endogenous cholesterol synthesis, as measured by hepatic and intestinal HMG-CoA reductase activities was reduced 15 and 17%, respectively, in oryzanol-fed animals following 8.5 wk dietary treatment (Table 2), these observations were not statistically significant ($P < 0.08$).

Experiment 2. Relative to the control animals, oryzanol-treated hamsters had reduced plasma TC (44%, $P < 0.01$), NON-HDL-C (57%, $P < 0.01$), and TG (46%, $P < 0.01$) (Table 3). Despite a 12% decrease in HDL-C, the oryzanol-treated animals maintained a more optimal NON-HDL-C/HDL-C profile (1.1 ± 0.4 vs. 2.5 ± 1.4 $P < 0.0075$). The body weights at the beginning and end of the experiment were 108 ± 4 (mean ± SEM) and 128 ± 4 g for the control group and 108 ± 5 and 128 ± 8 g for the oryzanol group, respectively.

TABLE 2
Cholesterol Absorption and HMG-CoA Reductase Activities in Liver and Small Intestines of Hamsters Fed Control and Oryzanol Diets in Experiment 1[a]

	Group	
Organs	Control	Oryzanol
Cholesterol absorption (%)	57.3 ± 2.1[a]	42.9 ± 2.0[b]
Liver HMG-CoA reductase (pmol/min per mg protein)	14.1 ± 0.7	12.0 ± 0.9
Small intestine HMG-CoA reductase (pmol/min per mg protein)	36.3 ± 3.8	30.4 ± 3.1

[a]Values are expressed as mean ± SEM ($n = 16$). Values not sharing a common superscript are significantly different at $P < 0.0001$. Measurements were taken 7 wk after the initiation of the control and oryzanol groups; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA reductase.

TABLE 3
Plasma Lipids, Lipoprotein Cholesterol and Fatty Streak Area in F_1B Hamsters Fed Control and Oryzanol Diets in Experiment 2[a]

	Group	
	Control	Oryzanol
TC	220 ± 29[a]	124 ± 14[b]
NON-HDL-C	156 ± 29[a]	67 ± 11[b]
HDL-C	65 ± 2	57 ± 4
TG	218 ± 41[a]	118 ± 10[b]
Fatty streak area ($\mu m^2/mm^2 \times 10^3$)	15 ± 3[a]	5 ± 1[b]

[a]Values are expressed as mean ± SEM (n = 10 for control and n = 9 for oryzanol—mg/dL). Values not sharing a common superscript are significantly different at $P < 0.01$, except TG, which are $P < 0.05$. See Table 1 for abbreviations.

DISCUSSION

The present studies were designed to investigate whether oryzanol, a unique constituent in the nonsaponifiables of RBO, is an active ingredient responsible, at least in part, for the hypocholesterolemic action of RBO. The results of experiment 1 showed that the addition of oryzanol (1%) to a hypercholesterolemic diet for 7 wk was associated with a 34% decrease in plasma NON-HDL-C level as compared to the controls. In experiment 2, in which oryzanol was added to a hypercholesterolemic diet at 0.5% (w/w) and fed for 10 wk, NON-HDL-C was reduced 57%. These two observations indicate that oryzanol is responsible, at least in part, for the observed cholesterol-lowering activity of RBO.

The cholesterol-lowering activity of oryzanol has been previously demonstrated by other investigators (12–17). Shinomiya *et al.* (15) showed that when 0.5 and 2.0% oryzanol were fed to rats on a high cholesterol diet (1.0% cholesterol + 0.5% cholic acid) for 4, 8, and 12 wk, plasma TC decreased on the fourth, eighth, and twelfth week in the group fed 2.0% oryzanol, and the eighth and twelfth week in the group fed 0.5% oryzanol. Plasma LDL-C and VLDL-C levels and liver cholesterol esters decreased in the group fed 2.0% oryzanol. In a study reported by Seetharamaiah and Chandrasekhara (16), 7 wk following oryzanol feeding, plasma NON-HDL-C levels significantly decreased in rats fed all levels of oryzanol (0.2, 0.5, 1.0, and 2.0%) as compared to the control group. When oryzanol was added to a cholesterol-free diet, no effects on plasma lipids or lipoproteins were observed, suggesting that oryzanol may affect cholesterol metabolism by altering dietary cholesterol absorption. One of the early studies with oryzanol reported that when oryzanol was fed to mice for 30 d (1 mg/day per kg body weight), it resulted in a decreased liver cholesterol biosynthesis as indicated by the lower ^{14}C-acetate or ^{14}C mevalonate incorporation into hepatic cholesterol. When oryzanol was injected (IP) to the mice, it caused an increased excretion of fecal sterols as indicated by the increased fecal excretion of total sterol-^{14}C and total bile acid-^{14}C derived from cholesterol-^{14}C injection (16). The cholesterol-lowering effect of oryzanol has also been reported in humans (12–14). When oryzanol was administered to patients with hyperlipidemia (type IIa, IIb, and IV) for 3 mon (300 mg/day), plasma TC and LDL-C levels decreased in Type IIa, IIb and type IV patients; oryzanol feeding also caused an increase in the plasma HDL-C levels in type II patients (12,13). In a study on chronic schizophrenic patients with dyslipidemia (TC ≥ 220 mg/dL, TG ≥ 150 mg/dL, or HDL-C ≤ 40 mg/dL), oryzanol feeding (3 × 100 mg/day) reduced plasma TC, LDL-C, and Apo B levels (14).

The cholesterol-lowering effect of RBO has been attributed to its higher nonsaponifiable content (1–3,5–8). A recent study by our laboratory has demonstrated that RBO prevents hypercholesterolemia induced by a high-cholesterol diet in hamsters by suppressing cholesterol absorption and enhancing fecal sterol excretion (7). It has been reported that feeding the nonsaponifiable lipids extracted from RBO in rats caused the increased fecal sterol excretion (2). The effect of nonsaponifiables, or oryzanol, on cholesterol absorption has not been previously reported. The present study has demonstrated that oryzanol feeding in hamsters caused a decreased dietary cholesterol absorption, and this suppressed cholesterol absorption was at least partially responsible for the lower plasma TC and NON-HDL-C levels induced by oryzanol feeding.

Oryzanol, which constitutes 20% of the nonsaponifiables of RBO, is a mixture of ferulic acid esters of plant sterols and triterpene alcohols. The mechanism by which oryzanol affects cholesterol absorption cannot be elucidated from our experiment. The mechanism by which plant sterols affect cholesterol absorption has been studied for many years but is still not fully understood. Several mechanisms have been hypothesized (27,29). These include: (i) plant sterols may form a nonabsorbable complex with cholesterol in the intestinal lumen; (ii) plant sterols may interact with and alter the size and/or stability of cholesterol-containing micelles in the intestinal lumen and thereby alter the efficiency with which cholesterol is delivered to the mucosal surface; (iii) plant sterols may interfere with cholesterol esterification in the mucosa cell; and (iv) plant sterols may interact with protein receptors on the mucosal cell surface which are necessary for cholesterol absorption. Unpublished results of our laboratory showing that one single oral bolus of oryzanol inhibits cholesterol absorption suggests that oryzanol interferes with cholesterol absorption at least by the mode of luminal action as well as or instead of intracellular events which reflect long-term effects of a feeding regimen. It is also possible that oryzanol exercises its effects on cholesterol metabolism at sites other than the intestine since other pharmacological responses of oryzanol, such as a growth-accelerating action, regulation of the estrous cycle, ability to promote skin capillary circulation (30), and antioxidant activity (31) have been reported.

The results in the oryzanol feeding experiments in the present studies showed that oryzanol induced lower cholesterol absorption without changing *de novo* cholesterol synthesis in the intestinal mucosa. This differs from that shown in the RBO feeding experiment in our previous study (7) in which

the lower cholesterol absorption in the RBO group was associated with a reciprocal increase in *de novo* cholesterol synthesis as indicated by the increased intestinal HMG-CoA reductase activity. This suggests that the other constituents in the nonsaponifiables of RBO may also exert certain effects on cholesterol metabolism in the intestinal mucosa.

In experiment 2 it was demonstrated for the first time that the oryzanol effects in plasma TC, NON-HDL-C, and cholesterol metabolism were associated with a significant decrease (67%) in early atherosclerosis. The simplest explanation for the decrease in macrophage-derived foam cells which constitute the aortic fatty streak area for the oryzanol group is the reduction in the NON-HDL-C fraction. However, other properties of oryzanol may be contributing to its antiatherogenic properties *via* other mechanisms. For example, the ferulic acid component of the oryzanol fraction has been shown to have antioxidant activity (32). Thus it is possible that the prevention of fatty streak formation in the oryzanol-fed hamsters could be associated with the inhibition of LDL oxidation by oryzanol, and in particular the ferulic acid component. The sensitivity of the hamster to the antioxidant activity of tocopherol and probucol as it relates to LDL oxidation and early atherosclerosis has recently been demonstrated (33). Finally, in light of the effects of RBO and the nonsaponifiables of RBO on fecal sterol excretion (2), it is plausible that oryzanol may alter the fecal sterol excretion in addition to its effect on cholesterol absorption.

The doses of oryzanol used in the first and second experiments, as a percentage of dietary energy, were approximately eightfold and fourfold that which could be obtained by an individual using physically refined rice bran oil as the major source of fat in their diet. These doses were used in order to study the mechanism(s) by which oryzanol affects cholesterol metabolism. Future studies could focus on the minimal amount of oryzanol needed for an optimal response.

In conclusion, this communication demonstrates that oryzanol, an unsaponifiable component of RBO, reduced plasma TC and NON-HDL-C by reducing cholesterol absorption. These properties were associated with a reduction in early atherosclerosis.

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Effect of Nicotine on Lipoprotein Metabolism in Rats

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ABSTRACT: Nicotine, a major component of cigarette smoke, plays an important role in the development of cardiovascular disease and lung cancer in smokers. The effect of nicotine on lipoprotein metabolism was studied using rats as the experimental animal. There was a significant increase in the total cholesterol, phospholipids, and triglycerides as well as the amount of lipids associated with very low density lipoprotein (VLDL) and low density lipoprotein (LDL) in sera of nicotine-treated rats. The incorporation of ^{3}H labeled leucine into the apo B was found to be increased both in the medium and associated cells in the hepatocytes isolated from nicotine-treated rats indicating an increased synthesis and secretion of the apo B containing lipoproteins. This was further confirmed by the higher incorporation of ^{14}C acetate into total and individual lipids of LDL and VLDL secreted into the medium as well as that associated with different lipids in the cell layer. The activity of lipoprotein lipase in extrahepatic tissues and plasma lecithin cholesterol acyl transferase activity were significantly lower in nicotine-treated rats. These results indicate that nicotine exerts hyperlipidemic effects particularly by increasing the synthesis and secretion of triglyceride-rich lipoproteins. Since nicotine is one of the major hazardous components present in cigarette smoke and tobacco, one can extrapolate that the deleterious effect exerted by nicotine on rats extends to cigarette smokers and those who use other forms of tobacco.

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Nicotine, one of the most pharmacologically active tobacco components, has a wide range of cardiovascular effects (1–4). Short-term experiments with rabbits (5) and humans (6) have shown that oral administration of the alkaloid raises plasma low density lipoprotein (LDL) cholesterol and lowers high density lipoprotein (HDL) cholesterol. These observations are clinically important because of the widespread use of smokeless tobacco products (7) and the increased prescription of nicotine-containing chewing gum in smoking cessation therapy (8). Treatment of 2 mg nicotine gum for 60 d in nonsmokers resulted in an increase in HDL and LDL cholesterol (9). Burch *et al.* (10) reported that the serum levels of total cholesterol triglycerides and LDL cholesterol were elevated in nicotine-treated rats. It appears that the accelerated synthesis of LDL through enhanced lipolytic conversion of precursor lipoproteins is one metabolic process responsible for creation of a lipoprotein profile by nicotine (11). Since liver is the major site of plasma lipoproteins, particularly very low density lipoprotein (VLDL) and LDL, we carried out some studies in isolated hepatocytes of nicotine-treated rats.

MATERIALS AND METHODS

Male albino rats (Sprague Dawley strain; age about 45 d, body weight 120–150 g) maintained on normal laboratory diet (Goldmohur rat feed) were used for these experiments. Water was provided *ad libitum*. The rats were divided into two groups of 15 rats each as follows: Group 1: control rats; Group 11: rats given nicotine. Nicotine 98% (Chemische Fabric CH-9470 Buchs, Fluka AG, Buchs, Switzerland) dissolved in physiological saline and pH adjusted to 7.2 with sterile 0.1 N HCl was administered orally for the experimental group. The daily dose of nicotine was 3.5 mg/kg body weight. The control rats received the same volume of physiological saline daily. The experimental period was 90 d.

Isolation of hepatocytes. Hepatocytes from nicotine-treated and control rats were isolated by collagenase perfusion according to the procedure by Seglen (12) and Sudhakaran *et al.* (13). Cells were suspended in Eagle's essential medium containing streptomycin (100 mg/mL), penicillin (100 mg/mL), insulin (0.6 mg/mL), and fetal bovine serum and were then plated in 35-mm plastic petri dishes. Cultures were maintained at 37°C in 95% air–5% CO_2 atmosphere. The medium was removed after 4–5 h, and the cell monolayer was washed with serum-free medium. The yield of hepatocytes from both control and nicotine-treated rat livers was comparable (control rat: 205×10^6 cells/liver, nicotine-treated rat: 200×10^6 cells/liver). More than 90% of the cells from both preparations were viable as assessed by trypan blue exclusion. The plating efficiency was also comparable. About 60% of the cells from both preparations attached to collagen coated overslips in 1 h after plating.

Metabolic labeling with ^{3}H leucine and ^{14}C acetate. The monolayer of hepatocytes was subsequently incubated with 2 mL of serum-free medium that contained radiolabeled and/or unlabeled metabolites. In radiolabeling experiments, ^{3}H leucine (30 μCi/mL) or ^{14}C acetate (10 μCi/mL) was added to each dish. Cells were incubated for different time intervals. At the end of the incubation period, the medium and cells were separately collected for lipoprotein analysis.

Isolation of lipoproteins. Lipoproteins, mainly VLDL, se-

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Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

creted into the medium by the cells were separated by preparative sequential ultracentrifugation according to the procedure of Havel *et al.* (14), in the presence of carrier serum in a Sorval ultracentrifuge (Dupont, Boston, MA).

Preparation of antisera against rat apo B. Rat serum VLDL and LDL were duplicated, 1 mL of VLDL or LDL was mixed with 1 mL of isopropanol at 4°C. It was then centrifuged and the supernatant discarded. The residue was again washed with isopropanol and dissolved in electrophoresis sample buffer and electrophoresed in a Hoeffer electrophoretic apparatus (San Francisco, California) at 30 mA for 8 h, using 7.0% SDS polyacrylamide gel electrophoresis according to Laemmli (15). The gel was then stained with Coomassie brilliant blue. The top-most band corresponding to apo B was then cut out, destained, and extensively washed with distilled water and dispersed finely using a hypodermic syringe in normal sterile saline. The suspension was mixed with Freund's complete adjuvant to get a fine emulsion. It was then injected subcutaneously into a rabbit at various sites. A booster dose was given on 14th d. A second booster dose was also given on 21st d (after 7 d). The animal was bled on 31st d (after 10 d of second booster), and antiserum was collected and dispensed as 0.5 mL aliquots in microcentrifuge tubes and kept at −20°C. The crossreaction of the serum with apo B was then checked by double diffusion technique using rat VLDL and LDL as antigens. The antiserum was found to be specific for rat serum apo B as it did not show any crossreaction with rat serum HDL, albumin, or human serum VLDL and LDL.

Immuno precipitation. Labeled apo B associated with lipoproteins was precipitated by adding anti-rat apo B serum, raised in rabbit. The antigen–antibody complex was collected by incubating with protein-A-Sepharose beads (16) and separated by SDS polyacrylamide gel electrophoresis (15). The gel slices were dissolved in 20% H_2O_2 and the radioactivity was measured using a scintillation counter.

Thin-layer chromatography and lipid analysis. Lipids associated with cells as well as VLDL and LDL were extracted by the method of Folch *et al.* (17). Individual lipids were separated by thin-layer chromatography (18) and fractions, mainly triglycerides, cholesterol, cholesterol esters and phospholipids, were scraped and quantitated by scintillation counting.

Cholesterol, triglycerides, and phospholipids were estimated in serum and tissues (19).

Other methods. Cellular protein was measured by the method of Lowry *et al.* (20). Rate of protein synthesis was determined by measuring the incorporation of ^{3}H leucine into trichloroacetic acid-insoluble materials using bovine serum albumin as carrier. Lipoprotein lipase (21) and lecithin cholesterol acyl transferase (22,23) were also assayed. Blood nicotine concentration was determined spectrophotometrically (24).

RESULTS

Concentration of lipids in serum and tissues. No significant change was noticed in gain in body weight of nicotine-treated rats (125 ± 4.2 g) when compared to control rats (135 ± 4.5 g).

Nicotine administration resulted in a significant increase in serum cholesterol, triglycerides, and phospholipids when compared to control group. Liver cholesterol, triglycerides, and phospholipids were also higher in nicotine-administered rats. Cholesterol, phospholipids, and triglycerides associated with the serum VLDL and LDL were also significantly higher in nicotine-treated rats (Tables 1 and 2)

Synthesis and secretion of apo B in hepatocytes in culture. In order to study whether the increased levels of VLDL and LDL observed in nicotine-treated rats were due to altered production of the lipoproteins in liver, hepatocytes isolated from animals were metabolically labeled with ^{3}H leucine and the apo B-containing lipoprotein secreted into the medium was analyzed. Primary cultures of hepatocytes derived from nicotine-treated rats showed increased incorporation of ^{3}H leucine into apo B-containing lipoproteins secreted into the medium, indicating increased synthesis and secretion of apo B (Fig. 1). The incorporation of radioactivity in cell-associated apo B was also higher in hepatocytes from nicotine-treated rats (Fig. 2). A pulse-chase experiment was carried out to find whether the rate of secretion of apo B-containing lipoproteins into the

TABLE 1
Concentration of Lipids in Serum and Tissues

	Serum (mg/100 mL)		Liver (mg/100 g tissue)	
Lipids	Control	Nicotine	Control	Nicotine
Cholesterol	60.52 ± 1.45	70.05 ± 1.69[a]	343.20 ± 7.55	454.10 ± 16.80[a]
Triglycerides	7.41 ± 0.17	10.21 ± 0.39[a]	445.20 ± 10.20	531.80 ± 18.08[a]
Phospholipids	128.20 ± 3.07	157.30 ± 6.13[a]	2212.00 ± 53.08	2690.00 ± 102.20[a]

[a]Values ± SEM of six experiments; *P* values superscript a <0.001.

TABLE 2
Concentration of Lipids in Serum Lipoproteins (LDL + VLDL) (mg/100 mL serum)[a]

Groups	Cholesterol	Triglycerides	Phospholipids
Control	14.60 ± 0.55	101.10 ± 2.38	2.01 ± 0.05
Nicotine	30.38 ± 1.10[a]	128.03 ± 5.20[a]	7.49 ± 0.29[a]

[a]Values ± SEM of six experiments; *P* values superscript a <0.001; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

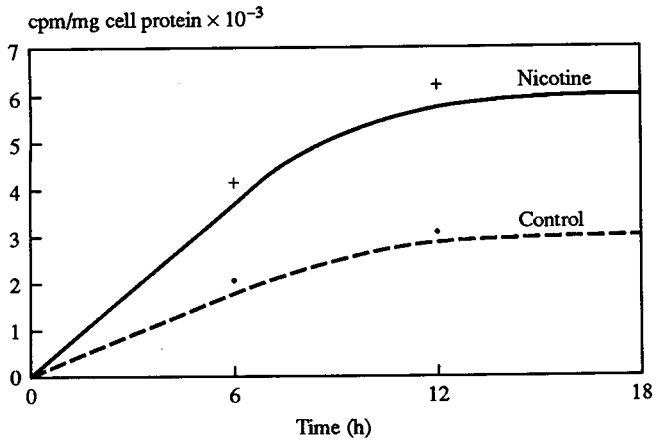

FIG. 1. Incorporation of ^{3}H leucine into apo B in medium.

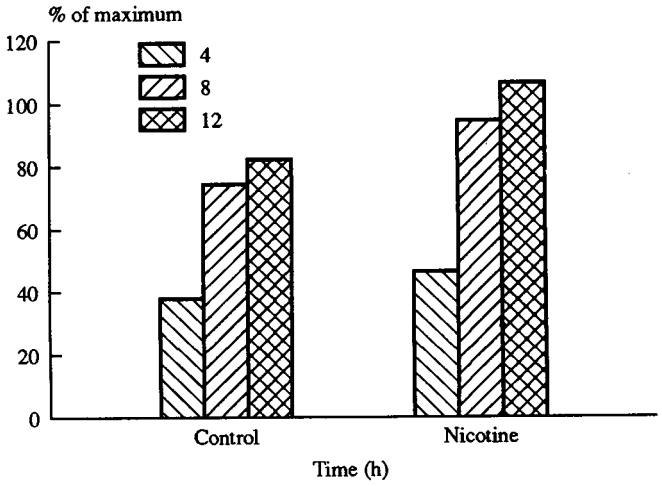

FIG. 3. Secretion of ^{3}H apo B (pulse chase experiment).

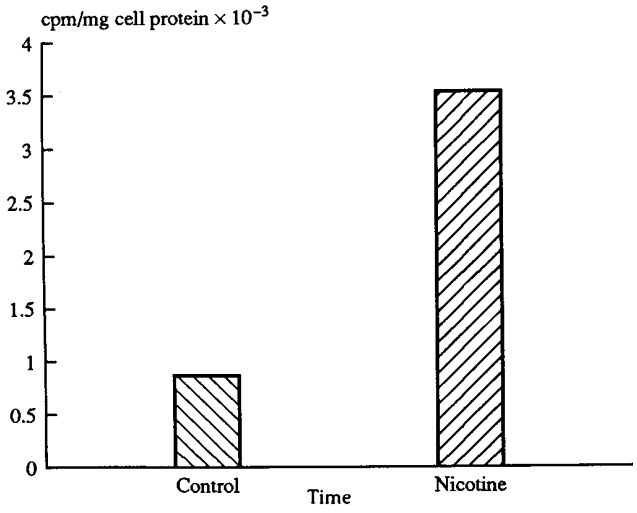

FIG. 2. Incorporation of ^{3}H leucine into apo B in cell layer.

medium was affected. The cells were preincubated in leucine-free medium for 1 h and were then pulse-labeled with 100 μCi of ^{3}H leucine for 3 h. Medium was removed and the cells were maintained in a medium containing nonradioactive leucine. The apo B secreted into the medium at different time intervals was immunoprecipitated and quantitated and expressed as the percentage of that secreted in 12 h in each case (Fig. 3). The fraction of the total ^{3}H-apo B released into the medium by hepatocytes isolated from nicotine-treated rats was significantly higher when compared to control rats. At 6 h nearly 60% of the total secreted apo B was released by hepatocytes derived from rats fed nicotine while control rat hepatocytes released 48%.

Incorporation of ^{14}C acetate into lipids associated with lipoproteins secreted by hepatocytes. The synthesis and secretion of lipoprotein by hepatocytes from nicotine-treated rats were also studied by metabolic labeling with ^{14}C acetate. Incorporation of ^{14}C acetate into cholesterol, phospholipids, triglycerides, and ester cholesterol of VLDL and LDL secreted into the medium was significantly higher in nicotine-treated rats when compared to control rats. ^{14}C acetate incorporation into cell-associated cholesterol, phospholipids, triglycerides, and ester cholesterol was significantly higher in the case of hepatocytes derived from nicotine-treated rats (Table 3).

Activity of lipoprotein lipase of extrahepatic tissue and plasma LCAT. Rats administered nicotine showed significantly lower lipoprotein lipase activity in aorta, heart, and adipose tissue when compared with control rats (Table 4).

Plasma LCAT activity was also decreased in nicotine-administered rats (Table 4).

Concentration of nicotine. The blood nicotine concentration in nicotine-treated rat was 10 μg/mL blood.

DISCUSSION

Nicotine has a definite effect on the concentration of lipids in different tissues, but the nature of the effect varies with the nature of the lipid and is different in different tissues. The reports on the effect of administration of nicotine are also not in agreement. Cluette Brown *et al.* (11) observed that oral administration of nicotine had no effect on plasma triglycerides, whereas it increased the cholesterol concentration. David and Turner (25) reported that nicotine caused an increase in the accumulation rate of triglycerides. Our findings, i.e., the increased concentration of different lipids in serum, is in agreement with the report of Booyse *et al.* (5) who observed a 26% increase in serum triglycerides and a 71% increase in total cholesterol by nicotine administration to rabbits and Lagrue *et al.* (9) who reported an increased level of plasma total cholesterol and triglycerides in nonsmokers following nicotine intake. The present study of serum LDL cholesterol confirms the earlier observation that nicotine-treated monkeys showed significantly higher levels of plasma LDL cholesterol (26).

TABLE 3
Incorporation of ^{14}C Acetate into Lipid Secreted into the Medium and Cell-Associated Lipids (cpm/mg protein)[a]

	Cholesterol	Triglycerides	Phospholipids	Ester cholesterol
Lipids secreted into the medium				
Control	411.0 ± 9.8	482.0 ± 13.0	1792.0 ± 43.0	375.0 ± 10.9
Nicotine	1016.0 ± 39.0[a]	2593.0 ± 98.0[a]	3898.0 ± 15.2[a]	902.0 ± 35.2[a]
Cell-associated lipids				
Control	823.0 ± 25.5	751.0 ± 18.0	811.0 ± 23.6	613.0 ± 14.7
Nicotine	3101.0 ± 117.8[a]	1593.0 ± 58.9[a]	1294.0 ± 50.4[a]	1122.0 ± 43.8[a]

[a]Values ± SEM of six rats; *P* values superscript a <0.001.

TABLE 4
Activity of Lipoprotein Lipase and Plasma LCAT[a]

	Lipoprotein lipase[b]			
Groups	Heart	Aorta	Adipose	Plasma LCAT[c]
Control	25.36 ± 0.96	80.15 ± 2.83	123.20 ± 3.44	160.30 ± 4.90
Nicotine	18.60 ± 0.39	65.31 ± 1.95	99.23 ± 2.68	133.40 ± 2.44

[a]Values ± SEM of six rats; *P* values superscript a <0.001.
[b]Glycerol μ mol liberated/h/g protein.
[c]Percentage increase in the ratio of ester cholesterol to free cholesterol during incubation; LCAT, lecithin cholesterol acyl transferase.

The increased incorporation of ^{3}H leucine into apo B associated with hepatocytes as well as that secreted into the medium suggests that synthesis and secretion of apo B, the major apoprotein secreted as VLDL and LDL, is higher in nicotine-treated rats. Results reported from our laboratory as well as those reported by others showed that hepatocytes in primary culture can synthesize both VLDL and LDL (27–31). Bell-Quinet and Forte (30) found that, apart from VLDL, some amounts of LDL can also be produced by liver cells in culture. It has been suggested that part of the VLDL at longer time intervals may be converted to LDL. Cluette Brown *et al.* (11) reported that chronic oral nicotine intake caused an elevation in both LDL protein and cholesterol suggesting that the alkaloid promotes an increase in the entire lipoprotein particles. Since a substantial amount of oral nicotine is absorbed in the intestine and then metabolized by hepatic microsomes (1,32), the alkaloid could enhance LDL synthesis by modifying hepatocyte smooth endoplasmic reticulum, where lipoproteins are manufactured prior to secretion. In this regard, chronic oral administration of nicotine increases hepatic microsomal enzyme system and stimulates protein synthesis (33). Increased incorporation of ^{14}C acetate into cholesterol and cholesterol esters associated with LDL and VLDL secreted by hepatocytes observed in the present study also indicates increased production of VLDL and LDL by hepatocytes from nicotine-treated rats.

Cellular cholesterol is a critical component in VLDL assembly. Any condition which causes an increase in cholesterol ester production is associated with an increased secretion of apo B as VLDL. A positive correlation between cholesterol ester content and apo B secreted as VLDL has been observed (31). Plasma lecithin cholesterol acyl transferase is the enzyme involved in the esterification of cholesterol. The esterification takes place on the surface of HDL. Both this enzyme and HDL are involved in the transfer of cholesterol from tissue to the liver for degradation. The increased concentration of cholesterol observed in nicotine-treated rats may be due to the decreased activity of this enzyme.

Increase in serum lipid level can also be due to decreased clearance of lipoproteins by extrahepatic tissues. Lipoprotein lipase is involved in the uptake of triglyceride-rich lipoproteins (chylomicrons and VLDL) by the extrahepatic tissues. Decreased activity of lipoprotein lipase in extrahepatic tissues in nicotine-administered rats may result in decreased uptake of serum VLDL, resulting in an increased levels of VLDL.

Cigarette smoking, chewing tobacco, and snuff using are the major forms of nicotine intake. The intake of nicotine per cigarette averaged 1.0 mg but ranged from 0.37 to 1.56 mg (34). A study of nicotine intake from smokeless tobacco reported an average of 3.6 mg nicotine from 2.5 g of oral snuff and 4.6 mg of nicotine from an average of 7.9 g of chewing tobacco, when both are kept in the mouth for 30 min (35). In our present work, the blood nicotine concentration in experimental animals was minute (10 μg/mL), but low-level nicotine caused significant changes in lipoprotein metabolism and produced atherosclerotic conditions. These results show the deleterious effect of nicotine in cigarette smokers and in users of smokeless tobacco.

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Non-Insulin Dependent Diabetes Mellitus in *Psammomys obesus* Is Independent of Changes in Tissue Fatty Acid Composition

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ABSTRACT: Recently it has been postulated that membrane fatty acid composition may be involved in the pathogenesis of insulin resistance and non-insulin dependent diabetes mellitus (NIDDM). The aim of this study was to determine whether alterations in tissue phospholipid (PL) fatty acids are present in hyperglycemic and hyperinsulinemic *Psammomys obesus*. On a native diet of salt bush, *P. obesus* (Israeli sand rat) remains lean and free of diabetes; however, when placed on a normal laboratory chow, a significant proportion of these animals develops a number of metabolic disorders associated with NIDDM, providing an ideal animal model of obesity and NIDDM. Four groups of mature *P. obesus* were studied: group A: normoglycemic and normoinsulinemic; group B: normoglycemic and hyperinsulinemic; group C: hyperglycemic and hyperinsulinemic; and group D: hyperglycemic and hypoinsulinemic. In liver and red gastrocnemius muscle, there were no significant differences between groups A, B, and C in fatty acid composition of PL. Minor differences in individual fatty acids were demonstrated in group D animals (increased liver 20:4n-6 and increased muscle 22:5n-3); however, the unsaturation indices in liver and muscle were not significantly different between any of the groups. In considering that the minor changes in group D animals were not demonstrated in hyperinsulinemic group B animals or hyperglycemic, hyperinsulinemic group C animals, it is likely that the differences in group D animals were secondary to the more severe disturbances in glucose homeostasis and hypoinsulinemia present in these animals. The results of this study suggest that in this rodent diabetic model significant disturbances in glucose homeostasis and hyperinsulinemia may develop independently of changes in tissue fatty acid composition.
Lipids 32, 317–322 (1997).

Recent interest has focused on the importance of tissue fatty acid phospholipid (PL) composition in the modulation of insulin action. A number of animal studies have examined the effect of different dietary fats on tissue fatty acid composition and correlated changes in fatty acid composition with insulin action (1,2), metabolic rate, and weight gain (3,4). These animal studies have also been recently supported by cross-sectional human studies where decreased insulin sensitivity was associated with a decreased concentration of polyunsaturated fatty acids in skeletal muscle PL (5), and a prospective study by Vessby and coworkers (6) in which individuals who later developed non-insulin dependent diabetes mellitus (NIDDM) had a higher proportion of saturated fatty acids in their serum cholesterol esters 10 years prior to the development of NIDDM. As a result of a number of these studies, it has been postulated that membrane fatty acid composition may be involved in the pathogenesis of insulin resistance and non-insulin dependent diabetes mellitus (5,7). However, it is not possible to separate secondary changes attributable to dietary factors from genetically determined differences in these studies. In an effort to examine this question further, we have utilized *Psammomys obesus*, an animal model of diabetes that naturally develops a range of defects in glucose metabolism and insulin action when fed standard laboratory chow (8). The aim of this study was to determine whether the development of hyperglycemia and hyperinsulinemia in *P. obesus* (Israeli sand rat) was accompanied by changes in the composition of membrane PL fatty acids in two important insulin-sensitive tissues—liver, and a representative skeletal muscle, red gastrocnemius. Impairment of insulin action in both the liver and skeletal muscle is central to the spectrum of metabolic disturbances observed in NIDDM in both rodent models (7,9) and humans (10). Membrane PL fatty acids were analyzed in four groups of animals studied: group A: normoglycemic and normoinsulinemic; group B: normoglycemic and hyperinsulinemic; group C: hyperglycemic and hyperinsulinemic; and group D: hyperglycemic and hypoinsulinemic.

MATERIALS AND METHODS

Animals. Mature, 18-wk-old male *P. obesus* ($n = 28$) (bred in Deakin University Animal House, Geelong, Victoria, Australia) were divided into four groups based on fed blood glu-

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Abbreviations: NIDDM, non-insulin dependent diabetes mellitus; PL, phospholipid.

TABLE 1
Body Weight, Muscle and Liver Lipid Content, and Glucose and Insulin Levels in *Psammomys obesus* Groups[a,b]

Group	Body weight (g)	Liver (% lipid)	Muscle (% lipid)	Glucose (mmol/L)	Insulin (μU/mL)
A ($n = 8$)	213.0 ± 11.0[c]	4.7 ± 0.4[c]	1.9 ± 0.1	4.7 ± 0.3[c]	55 ± 12[c]
B ($n = 7$)	253.4 ± 11.0[d]	7.2 ± 1.1[d]	2.1 ± 0.1	5.1 ± 0.5[c]	303 ± 46[d]
C ($n = 7$)	256.0 ± 13.4[d]	7.8 ± 0.9[d]	2.0 ± 0.2	11.2 ± 1.3[d]	474 ± 105[c]
D ($n = 6$)	216.3 ± 7.4[c]	4.4 ± 0.9[c]	1.7 ± 0.2	20.6 ± 2.1[e]	42 ± 8[c]

[a]Values are mean ± SEM for the four groups of *Psammomys obesus* described in text.
[b]Means within columns with different superscript letters are significantly different.
[c,d,e]($P < 0.05$).

cose and insulin levels measured sequentially at 4, 8, and 12 wk of age (see Table 1). Animals were housed in a temperature-controlled room (22 ± 1°C) with a 12-h light/dark cycle and were fed standard laboratory grain-based food (Barastoc, Victoria, Australia). The diet consisted of the following major fatty acids: 14:0 (1.1%), 16:0 (18.6%), 18:0 (1.9%), 18:1 (29.9%), 18:2n-6 (39.3%), 18:3n-3 (2.8%), 20:5n-3 (1.2%), and 20:6n-3 (2.2%). Blood samples were collected in the fed state for glucose and insulin measurements, and animals were anesthetized with an intraperitoneal injection of pentobarbitone sodium (60 mg/kg), then liver and red gastrocnemius muscle were quickly removed and stored at −70°C. Plasma insulin levels were measured in plasma using a double-antibody solid-phase radioimmunoassay (Phadeseph, Kabi Pharmacia Diagnostics, Uppsala, Sweden). Whole-blood glucose concentrations were measured enzymatically using a YSI glucose analyzer (model 1500 Sidekick; Yellow Springs Instrument Co., Yellow Springs, OH). All experiments were approved by the Animal Experimental Ethics Committee at Deakin University and complied with NHMRC of Australia guidelines for the care and use of laboratory animals.

Lipid extraction and fatty acid analysis. Lipids were extracted from liver and muscle with chloroform/methanol, followed by thin-layer chromatographic separation of the fatty acid classes to obtain the PL fraction (11). The total lipid content of the tissue was determined gravimetrically (11). The PL were subjected to saponification and methylation (11), and the resultant fatty acid methyl esters were separated by capillary gas–liquid chromatography (DANI 6500 HR, ICI International; Melbourne, Australia) on a 60 m × 0.32 mm i.d. Rtx-2330 crossbonded column of phase thickness 0.25 μm. Helium was used as the carrier gas with a flow rate of 43 cm^3/s. Cold on-column injection (60 to 260°C in 0.8 min) was used, and the flame-ionization detector temperature was 280°C. The column temperature was maintained at 95°C for the first 3 min, then increased at 10°C per min until 190°C was reached and maintained for a further 24 min until the final peaks were eluted. Individual fatty acid methyl esters were identified by comparison of their retention times with those of a standard fatty acid methyl ester mixture and by inclusion of an internal standard of diheptadecanoyl phosphatidylcholine in the aliquots prior to methyl ester formation. The results were corrected by use of response factors which were calculated using standard fatty acid methyl esters.

Statistical analysis. All results are expressed as mean ± SEM. Differences between groups were established using one-way analysis of variance. Fisher's least significant difference test was used to discriminate significant differences ($P < 0.05$) between groups.

RESULTS

Glucose and insulin. Table 1 describes mean glucose, insulin levels, and body weight for the four groups of *Psammomys obesus*. Animals were selected for each category on the basis of hyperglycemia being defined as whole-blood glucose levels of 8 mmol/L or more and hyperinsulinemia being defined as plasma insulin levels of 150 μU/mL as described previously (8). Group A animals were normoglycemic and normoinsulinemic at 18 wk and at all earlier times (glucose ≤8.0 mmol/L, insulin ≤150 μU/mL); group B animals at 18 wk were normoglycemic and hyperinsulinemic (glucose ≤8.0 mmol/L, insulin >150 μU/mL); group C animals at 18 wk were hyperglycemic and hyperinsulinemic (glucose >8.0 mmol/L, insulin >150 μU/mL), and group D were hyperglycemic and hypoinsulinemic (glucose >8.0 mmol/L, insulin ≤150 μU/mL) at 18 wk of age after displaying hyperglycemia and hyperinsulinemia at one or more of the earlier time points. The majority of animals that displayed either hyperglycemia or hyperinsulinemia at week 18 had similar glucose and insulin values at 12 wk of age. The body weights of groups B and C animals were heavier than animals in group A ($P < 0.05$).

Table 1 also describes the total percentage tissue lipid in groups A, B, C, and D animals. In the liver, groups B and C had a higher percentage lipid compared with group A ($P < 0.05$). Group D liver lipid levels were lower than group B and C and not significantly different from group A. In muscle, there were no significant differences in the lipid content between any of the four groups.

Fatty acid analysis. Tables 2 and 3 describe liver and muscle PL fatty acid composition for each group of animals fed standard laboratory food. In liver, there were no significant differences in the PL fatty acids between groups A, B, and C. The only significant differences were found in group D animals which had higher levels of 20:4n-6. No significant differences in the unsaturation index were found between groups A, B, C, or D. The n-6/n-3 ratio was similar in all

TABLE 2
Fatty Acid Profile of Liver Phospholipids[a,b]

Fatty acid	Group			
	A (n = 8)	B (n = 7)	C (n = 7)	D (n = 6)
(g/100 g fatty acids)				
16:0	17.1 ± 0.6	17.0 ± 0.6	15.9 ± 0.3	18.3 ± 1.1
16:1n-7	1.1 ± 0.1	1.2 ± 0.2	1.1 ± 0.1	0.8 ± 0.2
18:0	20.2 ± 1.3	21.1 ± 1.2	23.1 ± 1.0	18.1 ± 2.0
18:1	7.8 ± 0.6[c]	6.4 ± 0.5[c,d]	6.4 ± 0.4[c,d]	5.6 ± 0.5[c]
18:2n-6	21.2 ± 0.6	21.2 ± 1.0	21.3 ± 0.4	22.6 ± 0.7
20:3n-6	0.7 ± 0.0	0.8 ± 0.1	0.9 ± 0.0	0.7 ± 0.0
20:4n-6	7.2 ± 0.2[c]	7.0 ± 0.2[c]	6.7 ± 0.3[c]	9.1 ± 0.5[d]
20:5n-3	5.4 ± 0.6	5.1 ± 0.9	4.1 ± 0.4	4.1 ± 0.5
22:4n-6	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
22:5n-6	0.3 ± 0.0[d]	0.3 ± 0.0[d]	0.3 ± 0.0[d]	0.2 ± 0.0[c]
22:5n-3	1.8 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 0.2
22:6n-3	13.7 ± 1.0	14.8 ± 1.3	15.8 ± 0.5	15.2 ± 1.6
∑Saturated	37.3 ± 1.5	38.1 ± 1.0	38.9 ± 0.7	36.4 ± 1.1
∑Monounsaturated	8.8 ± 0.7	7.6 ± 0.6	7.4 ± 0.5	6.4 ± 0.6
∑n-6	29.6 ± 0.8[c]	29.3 ± 1.0[c]	29.5 ± 0.6[c]	32.8 ± 0.4[d]
∑n-3	20.9 ± 1.2	21.7 ± 1.8	21.7 ± 0.6	21.0 ± 1.2
Derived Indices				
20:4/20:3	10.0 ± 0.4[d]	8.7 ± 0.4[c]	7.8 ± 0.3[c]	13.1 ± 1.3[e]
n-6/n-3	1.5 ± 0.1	1.4 ± 0.2	1.4 ± 0.1	1.6 ± 0.1
20:4/20:5	1.5 ± 0.2	1.7 ± 0.3	1.8 ± 0.2	2.3 ± 0.2
20:4/22:5n-3	4.1 ± 0.4[c]	4.1 ± 0.2[c]	4.0 ± 0.2[c]	5.8 ± 0.6[d]
Unsaturation Index[f]	203 ± 7	205 ± 8	206 ± 3	212 ± 7

[a]Values are means ± SEM.
[b]Means within rows with different superscript letters are significantly different.
[c,d,e]$P < 0.05$.
[f]Unsaturation index = average number of double bonds per fatty acid residue × 100.

groups; however, group D animals had a significantly higher ratio of 20:4n-6/20:3n-6 and 20:4n-6/22:5n-3. In muscle there were no significant differences in the PL fatty acid composition between groups A, B, or C. However, group D compared with groups A, B, and C had significantly higher levels of 22:5n-3 and lower 20:5n-3. No significant differences were demonstrated in the unsaturation index between any of the groups. The ratio of 20:4n-6/20:5n-3 was higher in group D animals than groups A and B, but not group C. Total monounsaturated fatty acid levels were significantly lower in group D animals than groups B and C, although not group A.

DISCUSSION

The results of the current study clearly demonstrate that hyperinsulinemia and hyperglycemia can develop in animals independently from major alterations in membrane PL fatty acid composition.

Psammomys obesus is an ideal animal model in which to examine the development of NIDDM. These animals display a heterogeneous development of hyperinsulinemia and hyperglycemia when consuming standard laboratory food and can be divided into four groups (A, B, C, D) based on glucose and insulin levels as previously described (8). Group A animals are normoglycemic and normoinsulinemic, group B are normoglycemic and hyperinsulinemic, group C are hyperglycemic and hyperinsulinemic, and group D are hyperglycemic and hypoinsulinemic. In previous studies we have demonstrated that group C animals, when compared with group A animals, have increased body weight, increased epididymal and perirenal fat stores (12,13), and significant hyperphagia (12,13). From the current study, it is clear that metabolic changes in group C animals were not accompanied by any significant differences in individual tissue PL fatty acids when compared with group A or B animals. In addition, individual tissue fatty acid compositions were similar between all groups as was the unsaturation index and n-6/n-3 ratio of fatty acids.

On the other hand, group D animals, which develop severe diabetes and are hyperglycemic and hypoinsulinemic, demonstrated some differences in PL fatty acid composition, including increased 20:4n-6 in liver and increased 22:5n-3 in muscle. These animals also demonstrated an increased 20:4n-6/22:5n-3 ratio in liver and 20:4n-6/20:5n-3 in muscle. Despite these relatively minor changes, no significant differences were apparent in the unsaturation index of liver or muscle in group D animals compared with A, B, or C. In considering the relatively minor fatty acid differences in group D animals, and the fact that such changes were not demonstrated in hyperinsulinemic group B animals or hyperglycemic, hyperinsulinemic group C animals, it is most likely that the fatty acid changes in the group D animals were secondary to the more severe disturbances in glucose homeostasis and hypoinsulinemia.

TABLE 3
Fatty Acid Profile of Red Gastrocnemius Phospholipids[a,b]

Fatty acid	Group A (n = 8)	B (n = 7)	C (n = 7)	D (n = 6)
(g/100 g fatty acids)				
16:0	19.0 ± 0.5	20.3 ± 0.6	20.5 ± 0.5	19.2 ± 0.7
16:1n-7	1.1 ± 0.2	1.2 ± 0.2	1.2 ± 0.1	0.7 ± 0.2
18:0	11.6 ± 1.1	11.4 ± 1.2	11.7 ± 1.0	10.4 ± 1.2
18:1	7.1 ± 0.2	7.9 ± 0.4	7.4 ± 0.2	6.7 ± 0.1
18:2n-6	17.2 ± 0.3	16.4 ± 0.7	17.6 ± 0.7	18.9 ± 0.7
20:3n-6	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
20:4n-6	6.2 ± 0.3	6.1 ± 0.3	5.6 ± 0.2	5.7 ± 0.2
20:5n-3	3.5 ± 0.5[e]	3.1 ± 0.5[d,e]	2.3 ± 0.2[c,d]	1.9 ± 0.2[c]
22:4n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
22:5n-6	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
22:5n-3	3.2 ± 0.1[c]	3.4 ± 0.2[c]	3.6 ± 0.2[c]	4.9 ± 0.3[d]
22:6n-3	24.8 ± 1.0	25.8 ± 1.4	24.8 ± 0.9	24.5 ± 1.1
∑Saturated	30.6 ± 1.1	31.8 ± 1.2	32.2 ± 0.8	29.6 ± 0.6
∑Monounsaturated	8.2 ± 0.2[c,d]	8.3 ± 0.3[d]	8.6 ± 0.3[d]	7.5 ± 0.3[c]
∑n-6	24.5 ± 0.2	23.5 ± 0.7	24.2 ± 0.6	25.7 ± 0.5
∑n-3	31.5 ± 0.6	32.2 ± 1.8	30.6 ± 1.1	31.2 ± 1.3
Derived Indices				
20:4/20:3	9.9 ± 0.4	10.3 ± 0.7	9.2 ± 0.4	9.8 ± 0.5
n-6/n-3	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
20:4/20:5	1.9 ± 0.2[c]	2.2 ± 0.2[c]	2.6 ± 0.2[c,d]	3.2 ± 0.3[d]
20:4/22:5n-3	2.0 ± 0.2[d]	1.9 ± 0.2[d]	1.6 ± 0.1[d]	1.2 ± 0.0[c]
Unsaturation Index[f]	254 ± 4	256 ± 9	248 ± 5	253 ± 7

[a]Values are means ± SEM.
[b]Means within rows with different superscript letters are significantly different.
[c,d,e]$P < 0.05$.
[f]Unsaturation index = average number of double bonds per fatty acid residue × 100.

This study presents the first report of PL fatty acid profile of this novel rodent model of diabetes. Interestingly, *P. obesus* individuals have tissue membrane 20:4n-6 levels less than half of values reported in several rodent species (14,15). Furthermore, tissue membrane 22:6n-3 levels, particularly in the red gastrocnemius, were greater than double those reported for other rodents (14,15).

In the presence of diabetes, most studies in rodents, although not all (16), have reported reduced 20:4n-6, and increased or unaltered 18:2n-6 content in tissue and membrane PL fractions (15,17,18). These alterations are consistent with the insulin-sensitive nature of the Δ-6 and Δ-9 desaturase enzymes, with reduced activity present as a consequence of insulin insufficiency or severe insulin resistance (19). In the diabetic group D of *P. obesus,* the absence of significant alterations in both 20:4n-6 and 18:2n-6 in the red gastrocnemius muscle and the elevation in 20:4n-6 in the liver would suggest that in these diabetic animals, sufficient insulin was available to prevent an impairment of the Δ-6 and Δ-9 desaturase activity, despite the failure of the circulating insulin levels to maintain normoglycemia. However, it should be noted that in insulin-deficient streptozotocin diabetic rats the altered fatty acid profile of the major PL was varied, with phosphatidylcholine and phosphatidylinositol most susceptible to the diabetes-induced alterations (15). The differential alterations in fatty acid composition of the major PL classes suggest that the evaluation of total PL fatty acid composition, as analyzed in the current study, may fail to detect pronounced fatty acid changes in one or more PL classes. Similarly, PL isolated from differing tissues (20) and subcellular membrane fractions may demonstrate varied responses prior to and during the development of diabetes. Yet despite these potential concerns, the absence of significant alterations in total membrane PL fatty acids in the insulin-resistant group B and group C *P. obesus* suggests that a specific defect in membrane fatty acid composition is unlikely to be central to the development of insulin resistance in this rodent model. Moreover, in the absence of specific alterations in fatty acid composition consistent with insulin deficiency in the diabetic group D animals, the changes in membrane fatty acid composition are likely to occur as a consequence of the altered metabolic state. The secondary nature of these changes is consistent with previous reports on diabetic rodents in which insulin treatment ameliorates many of the alterations in membrane PL fatty acids (21,22)

Although not directly examined in this study, disturbances in fat metabolism may underly the development of hyperglycemia. Group C animals are hyperinsulinemic, hypertriglyceridemic, have increased body weight, increased fat stores (8,12,13), and in the present study, a significant increase in liver fat accumulation suggesting a significant disturbance in energy balance and fat metabolism. It is possible that these disturbances in fat metabolism interfere with glucose metabolism, as first suggested by Randle and coworkers (23), and contribute to the hyperglycemia demonstrated in group C animals. Recent evidence

also suggests that fat accumulation may contribute to the pancreatic defect that results in hypoinsulinemia (24). It is then, only after the development of severe insulin resistance with hyperglycemia and hypoinsulinemia, that secondary changes in PL fatty acid composition occur as seen in group D animals.

In contrast to studies in animal models fed standard diets, dietary intervention studies have clearly demonstrated the effectiveness of different dietary fats in altering membrane PL fatty acid composition and subsequent effects on insulin action. A positive relationship has been described between membrane PL long-chain n-3 fatty acid content and muscle insulin action (25,26); when diets high in saturated fat were supplemented with long-chain polyunsaturated n-3 fatty acids, the resultant increase in the unsaturation content of membranes correlated with improved insulin sensitivity (27). These dietary intervention studies have been supported by *in vitro* studies which have demonstrated that increased levels of polyunsaturated fatty acids increased membrane fluidity (28), and insulin action (1,29,30). It is clear from these studies that altering the membrane fatty acid composition (either *in vivo* or *in vitro*) will be accompanied by changes in insulin action, and it is possible that the metabolic effects of different dietary fatty acids may in fact be mediated *via* alterations in membrane composition. In a cross-sectional human study, Bjorkman and coworkers (5) examined the relationship between the fatty acid composition of skeletal muscle PL and insulin sensitivity in normal subjects and demonstrated that decreased insulin sensitivity was associated with decreased concentration of polyunsaturated fatty acids in skeletal muscle PL of normal men. In addition, a recent longitudinal study by Vessby and coworkers (6) demonstrated significantly increased saturated fatty acids in the serum cholesterol esters of subjects who developed NIDDM compared with those who remained normoglycemic. As discussed by these workers (5,6), the degree to which these changes reflect environmental differences rather than genetic predisposition is unclear. Clearly, it is possible that dietary fats, *via* modification of membrane PL fatty acids, play an important role in the development of insulin insensitivity. However, in *Psammomys obesus*, a genetically susceptible rodent model of diabetes, no significant changes in liver or red gastrocnemius muscle membrane PL were present despite hyperinsulinemia and/or hyperglycemia in the group B and C animals. With severe diabetes (group D), significant alterations in PL fatty acid composition were measured although these changes were inconsistent with previous reports in diabetic rodent models. From the results of this study, it can be postulated that the changes in total membrane PL fatty acids may reflect environmental differences, as a consequence of diabetes, with significant defects in insulin sensitivity and glucose metabolism potentially developing independent of the membrane fatty acid composition.

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A Rapid Method for the Determination of Vitamin E Forms in Tissues and Diet by High-Performance Liquid Chromatography Using a Normal-Phase Diol Column

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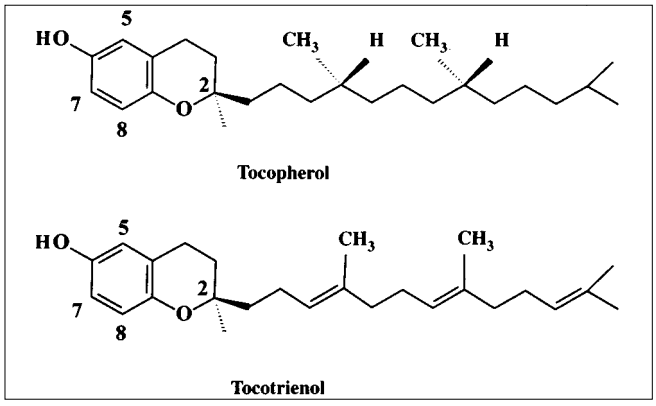
ABSTRACT: This paper describes a simple method for the analysis of tocopherols in tissues by which frozen tissues –70°C were pulverized at dry ice temperatures (–70°C) and immediately extracted with hexane. There was no need to remove the coeluting lipids from tissues by saponification, since at that level of neutral lipids in the sample, there was no reduction in fluorescence response. For the analysis of oil, in which large amounts of neutral lipids were coextracted, a 20% reduction of fluorescence response was observed, but the response was equal for all tocopherol forms, and was appropriately corrected. Saponification was used only when tocopherol esters were present, and only after an initial hexane extraction to remove the free tocopherols in order to avoid their loss by saponification, particularly non α-tocopherol and tocotrienols. All the tocopherols and tocotrienols were separated on a normal-phase diol (epoxide) column that gave consistent and reproducible results, without the disadvantages of nonreproducibility with silica columns, or the lack of separation with reversed-phase columns. The tocopherols were quantitated by using a tocopherol form not present in the sample as an internal tocopherol standard, or using an external tocopherol standard if all forms were present, or when the sample was saponified. Piglet heart and liver samples showed the presence of mainly α-tocopherol, with minor amounts of β- and γ-tocopherol and α-tocotrienol, but no δ-tocopherol. Only small amounts of tocopherol esters were present in the liver but not in the heart.

Lipids 32, 323–330 (1997).

Vitamin E has received much attention recently because of its antioxidant activity and its possible preventative role in degenerative disease processes. "Vitamin E" is a generic term which includes a mixture of tocopherols (T) and tocotrienols (T_3) which differ in the number and position of methyl groups on the fused chromanol ring, and the absence or presence of three double bonds in the isoprenoid side chain. The major naturally occurring tocopherol forms are 5,7,8-trimethyltocol (α-T), 5,8-dimethyltocol (β-T), 7,8-dimethyltocol (γ-T), and 8-methyltocol (δ-T), and the corresponding tocotrienols are 5,7,8-trimethyltocotrienol (α-T_3), 5,8-dimethyltocotrienol (β-T_3), 7,8-dimethyltocotrienol (γ-T_3), 8-methyltocotrienol (δ-T_3) and no methyl groups—tocol (Scheme 1).

In general, the current procedures for the analyses of tocopherols in tissues involve the removing of the tissue from the animal, analyzing it fresh, or more often, storing the sample at –70 to –80°C until it is analyzed, followed by thawing and mechanically homogenizing the sample (1–14). After addition of ethanol, the homogenate is extracted with hexane, or acetone (1,2,6,13) and analyzed directly for tocopherols (1,2,4,9–11,13), or saponified with alkali to remove the coextracted neutral lipids prior to analysis (3,5–8,12,14). Saponification has been recommended by some for tissue analyses (3,8), despite the fact that it causes extensive loss of all tocopherols (7,15), and tocotrienols (16), and that the loss is different for the various forms (7,15).

The analyses of tocopherol in foods and feeds (1,8,9,14,15, 17–20), oils and seeds (1,19,21–27), plasma (2,4,6–10, 13–15,28-32), platelets (30), and red cells (4,7,28,32) are the



SCHEME 1

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Abbreviations: CP, cross polarization; HPLC, high-performance liquid chromatography; MAS, magic angle spinning; NMR, nuclear magnetic resonance; T, tocopherol; T_3, tocotrienols; TAG, triacylglycerol.

same as for tissues, except for the prior homogenization step. Foods or feeds are ground and extracted directly (1,9) or saponified and extracted (1,8,14,15,17–20), vegetable oils are directly extracted (21–25,27) or saponified and then extracted (19,25,26), and plasma, platelets and red cells are treated with ethanol (2,6–10,13–15,28–31) or detergents (4,32) to remove proteins by precipitation, followed by hexane extraction.

The tocopherols are separated by high-performance liquid chromatography (HPLC) on normal-phase (1,3–5,8–11,15, 18,19,21–23,25–27), or reversed-phase columns (2,6–8, 12–14,17,19,20,24,28–32). A normal-phase column, bonded with amino–cyano groups, was used successfully to separate all vitamin E forms (9,19,23). Generally, tocopherols and tocotrienols are detected by fluorescence, although ultraviolet (3,13,19,28) or evaporative light-scattering detection (24,25) has also been used. However, these latter detectors are not selective (19,21) and less sensitive (19,21,25).

In this communication, a simplified method is presented which avoided the thawing and mechanical homogenization step that may cause loss of tocopherols. There was no need for added antioxidants to minimize loss of tocopherols. Direct injection of the hexane extract and detection by fluorescence was shown to be quantitative, and the various forms were not affected differently by the presence of triacylglycerol (TAG) in the mixture. Furthermore, a diol column was used for the separation of all eight tocopherol and tocotienols. The "diol" column, which is really an "epoxide" column, had the advantage of both giving more reproducible and consistent separations than silica columns (15,19,23) and separating all forms, unlike reverse-phase columns (7,8,19,20,28,31)

MATERIALS AND METHODS

Materials. Solvents were HPLC grade (Fisher Scientific, Nepean, Ontario, Canada) except absolute ethanol. The tocopherol forms α-, β-, γ- and δ-tocopherol (T), and 5,7-dimethyltocol (5,7-T; synthetic) were purchased from Matreya, Inc. (Pleasent Gap, PA). α-T_3 isolated from oats was a gift from Dr. Bill Collins (Center for Food and Animal Research, Ottawa, Ontario, Canada). Red palm oil, an enriched tocopherol and tocotrienol fraction from palm oil (Nutrolein, Golden Palm Olein) was a gift from the Malaysian Palm Oil Council of America, Inc. (Chicago, IL). Triolein was obtained from Nu-Chek-Prep, Inc. (Elysian, MN).

Tissue preparation. Heart and liver samples were taken from experiments in which newborn piglets were fed milk replacer diets for 4 wk as previously described (33). All the milk replacer diets contained additional α-T succinate (100 IU per kg diet) besides the natural tocopherols and tocotrienols in the dietary ingredients. The lactation and piglet starter diets were prepared to contain vitamin E acetate at 54 and 81 IU/kg, respectively.

The tissues were removed from the piglets at the end of the experiment and immediately frozen between blocks of dry ice and stored at −70°C until analyzed. The same general areas of the liver and heart were selected by the pathologist for vitamin E analysis. All operations for vitamin E analyses were carried out in a low-light environment. Two separate samples of approximately 1 g tissue were accurately weighed in the frozen state on a watch glass (cooled on dry ice) and immediately pulverized using a steel mortar and pestle at dry ice temperature, as described previously (34). The pulverized tissue was placed into 50-mL conical tubes with cap (Falcon™ polypropylene tubes; Becton Dickinson Labware, Franklin Lakes, NJ) containing 2 mL of water chilled in an ice bath. Polypropylene tubes were used for safety reasons during sonication. Five mL of cold absolute ethanol was added immediately to wash down any sample that stuck to the sides of the tube. In addition, 4 μg of one of the tocopherols was added as internal standard to each tube. (For choice of internal standard, see below.) The samples were sonicated (Model W-225; Heat Systems-Ultrasonic Inc., Farmingdale, NY) at 50% power setting for 45 s while the tubes were maintained in an ice bath. The sonication probe was rinsed with an additional 5 mL of cold absolute ethanol. Ten mL of ice-cold hexane was added to the tube, and the contents were mixed with a vortex for 30 s. The samples were centrifuged at 2500 rpm for 15 min at 0°C, after which 5 mL of the hexane layer was removed (to avoid picking up some of the lower phase) into a 100-mL round-bottom flask. The hexane was removed by flash evaporation using a rotary evaporator, then immediately taken up in 1 mL of hexane, and transferred to a 1.5-mL amber HPLC vial. The flask and vial were flushed with nitrogen. The vials were stored at −70°C until analyzed by HPLC, usually the same day. Samples were occasionally tested a week later and the results were found to be the same.

Extraction of tocopherols from diets and oils. If tocopherol esters were present in the diet, a modified saponification step was used. Ten mL of cold ethanol and an internal tocopherol standard was added to about 1 g of diet. The samples were sonicated, mixed using a vortex, and extracted with three portions of 10 mL of hexane to remove all TAG and vitamin E. The hexane layers were combined, reduced in a rotary evaporator, taken up in 1 mL of hexane, and analyzed. This fraction represented the free tocopherols in the diet. To the remaining ethanol mixture, 100 mg of ascorbic acid, 2 mL of a 60% KOH (wt/v), but no internal standard were added, and the solution was heated at 70°C for 10 min. The sample was then reextracted with three portions of 10 mL of hexane. The hexane extract could be washed with water to remove any traces of KOH. The hexane layers were combined, reduced, and taken up in 1 mL of hexane and analyzed. This fraction represented the esterified tocopherols in the diet, and it was quantitated with α-T as the external standard, since α-T esters are the usual form of vitamin E added to diets. Tocopherols and tocotrienols were directly extracted from oils with hexane after addition of ethanol and an internal tocopherol standard.

HPLC method. Tocopherols were analyzed by an HPLC (Model 1090, Hewlett-Packard, Palo Alto, CA) equipped with an autosampler (25 μL syringe), a fluorescence detector (Model FL2000; Spectra-Physics Analytical, Fremont, CA), and a data acquisition system (HP 3365 Series II ChemSta-

tion). A normal-phase Supelcosil LC-Diol column (25 cm × 4.6 mm, 5 μ particle size; Supelco Inc., Bellefonte, PA) was used, operated at room temperature, with hexane/propan-2-ol (99:1) as the mobile phase for 25 min, at a flow rate of 1 mL/min. The solvent mixture was prepared fresh each day and sparged with helium for 10 min. Continuous sparging with helium led to an increase in retention time of δ-T from 16 to 21 min over a 6-h period. This was due to a reduced content of propan-2-ol in the mixture from 1.00 to 0.61% during 6 h sparging, as measured by gas–liquid chromatography. The reason was that hexane/propan-2-ol formed an azeotropic mixture (77:23) which was selectively removed during sparging because of its lower boiling point at 62.7°C (35). This decrease of propan-2-ol lowered the polarity of the solvent, and therefore increased the retention times of the tocopherols. To avoid this annoyance, sparging was discontinued after 10 min, since sparging is not critical for fluorescence detection. The fluorescence detector excitation wavelength was 296 nm with an emission wavelength of 330 nm.

Quantitation. The samples were quantitated using selected tocopherol forms both as internal and external standard. The choice of internal standard depended on which tocopherol was absent in the samples to be analyzed. Liver and heart tissues, as well as red palm oil, contained no δ-T, which was therefore used as the internal standard. The dietary oils contained no α-T_3, and therefore, 5,7-T (a commercial product) was chosen as the internal standard, because on this column, α-T_3 did not separate from 5,7-T. An external standard was selected when all forms of vitamin E were present in the sample to be analyzed, or when a sample was saponified. α-T was the external standard of choice, because it is the major form of vitamin E present in biological samples, and the usual form added to diets. To measure the recovery, the detector response of the internal standard was compared to identical amounts of the same tocopherol measured externally. The average recovery of tocopherols was found to be 95 ± 7% (n = 10). The fluorescence detector was tested for linearity over the range from 10 to 100 ng for all vitamin E forms.

Measurement of nuclear magnetic resonance (NMR) spectra. The solid state ^{13}C CP/MAS NMR (cross polarization, magic angle spinning, NMR) spectra were recorded on a modified NT-270 Nicolet NMR spectrometer (Madison, WI). Spectra were obtained in natural abundance at a frequency of 68.055 MHz. CP/MAS were performed using a multinuclear CP/MAS probe equipped to accommodate 7.0-mm diameter spinners. MAS was carried out at 4.0 KHz. The magic angle was adjusted using the aromatic resonance line of the ^{13}C CP/MAS spectrum of hexamethylbenzene. ^{13}C spectra were acquired with a contact time of 2 ms, high power proton decoupling during acquisition, a recycle delay of 3 s, and a spectral width of 20 KHz. Typically, 10,000 transitions were accumulated. The chemical shifts were referenced to tetramethylsilane.

Statistics. All data were analyzed using the general linear models procedure, and the mixed models procedure of SAS (36), with terms for litter, diet, litter by diet interaction, animal variation within litter by diet, and variations between determinations. Where the variance component was zero for litter by diet interaction, this term was pooled with the "among piglets" term to test for diet differences.

RESULTS AND DISCUSSION

Pulverization and extraction step. This method was developed to avoid the thawing and mechanical homogenization step presently used in the analyses of vitamin E (1–14). Generally, tissues are removed immediately from experimental animals and stored at −70 to −80°C until analyzed. The technique described here maintains the tissues at that temperature (−70°C) during the weighing and pulverization step. The pulverized sample was then transferred directly into tubes containing water and ethanol at 0°C, and sonicated to provide dispersion of the tissue, before extraction of vitamin E with hexane. The addition of ascorbic acid or pyrogallol was not found to be necessary to protect vitamin E against degradation during the pulverization at −70°C. We have previously reported the same pulverization method at dry ice temperature for the extraction of intact total lipids from tissues and found that it protected lipids from lipolysis and oxidation (34).

Is saponification necessary? The extraction of vitamin E from tissues with hexane coelutes neutral lipids which interfered with the identification of the vitamin E forms, if ultraviolet (13,21,28,31) or evaporative light-scattering detectors (24,25) were used. These interfering lipids were removed by saponification, but this step reduced the recovery of all tocopherols, and in particular, δ-T (7,8,15,16,20,23) and α-T_3 (16). The reduction was dependent on the concentration of KOH (15), the temperature of saponification [room temperature (15,20) vs. 70°C (7,8,16)], and the TAG content (7). Despite this inherent problem, the saponification step is still recommended (3,7,8,15,19,20,21). The use of fluorescence detection should not cause interference, since this detector is specific for the chromanol chromaphore found in vitamin E. Even though several authors have demonstrated that tocopherols were measured successfully in the presence of neutral lipids (1,21,23,25,27), there seems to be a reluctance to delete the saponification step. A systematic evaluation was therefore undertaken to determine the effects of TAG on the quantitation of the tocopherols and tocotrienols using fluorescence detection (see section below). To reduce the neutral lipid content particularly from oils and oil seeds, some have suggested methanol extraction (27) or 1:10 dilutions of the hexane extract (24).

Separation of the tocopherols on a normal-phase diol column. The diol column gave consistent and reproducible separations of all four tocopherols (α-T, β-T, γ-T, and δ-T) and four tocotrienols (α-T_3, β-T_3, γ-T_3, and δ-T_3). Figure 1 shows a separation of a mixture of the four tocopherol standards and an extract from red palm oil. Red palm oil was chosen because it is known to contain all four tocotrienols (24), which are not commercially available. 5,7-Dimethyltocol, although commercially available, was of limited value as an internal

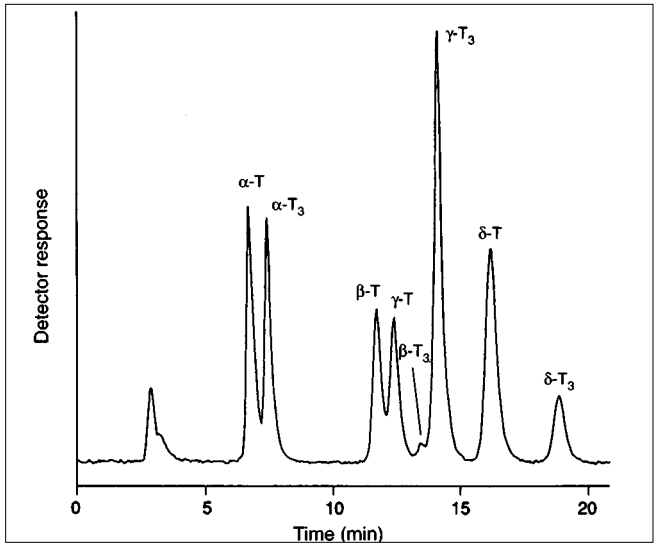
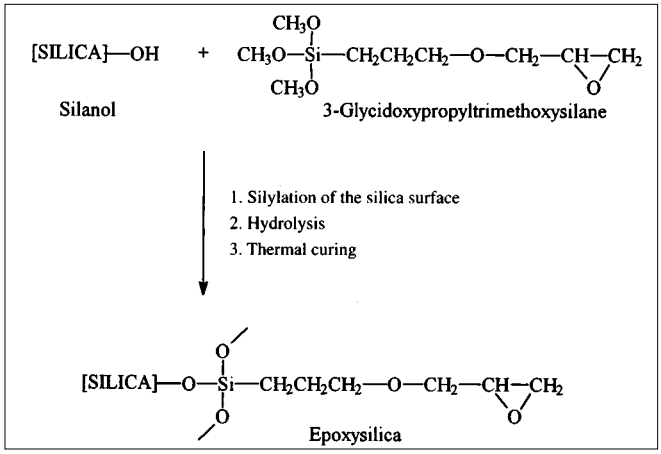


FIG. 1. Separation of tocopherol (T) and tocotrienol (T_3) by high-performance liquid chromatography on a normal-phase Supelcosil LC-diol column (Supelco Inc., Bellefonte, PA) using hexane/propan-2-ol (99:1) as the mobile phase and a flow rate of 1 mL/min.



SCHEME 2

standard, since it had the same retention time as α-T_3 that is generally found in small amounts in tissues. Tocol (for structure see Scheme 1), not commercially available, would have been a better choice as a general internal standard, because it is not present in tissues or oils, and it separated well from all tocopherols and tocotrienols on normal-phase columns as reported by some investigators (3,7).

The diol column behaved as a normal-phase column separating all the eight forms of vitamin E, as expected using silica-based columns (1,7,8,15,18,19,21–23,25,27). However, silica columns were reported to be unstable, occasionally yielding poor reproducibility (8,15,19,23,25), as experienced in our laboratory. For this reason, Rammell and Hoogenboom (23) proposed the use of an amino–cyano polar phase column in which cyano and amino groups were bonded to the surface hydroxyl groups of silica. This resulted in greater reproducibility and improved resolution of all tocopherols and tocotrienols. However, the amino group ionized with use of polar organic solvents, and caused increased retention times and peak broadening. This problem was eliminated by incorporating formic acid into the eluate. However, the authors expressed concern that the stability of the column may be affected by continued use of formic acid (23). On the other hand, the diol column has no ionizable groups.

The diol column was prepared by reacting 5 μm spherical silica particles containing surface silanol (Si-OH) groups with 3-glycidoxypropyltrimethoxysilane (Scheme 2) to furnish a bonded phase with a ligand density of 3.8 μmoles/m^2 on the silica. Evidence for the intact epoxy ring system on the bonded phase was provided by solid-state ^{13}C CP/MAS NMR of the packing material, which exhibited signals at δ 7.8 ppm (Si–*C*H_2), δ 23.4 ppm (Si–CH_2*C*H_2), δ 42.3 ppm (O–*C*H_2–CH of epoxy ring), δ 49.8 ppm (C*H*$_2$–O– of epoxy ring), and δ 70.9 ppm (double intensity, *C*H–O of epoxy ring plus Si–CH_2CH_2*C*H_2–O). If the epoxy ring had cleaved into a diol, the signal at δ 42.3 ppm would be absent and the intensity of the signal at δ 70.9 ppm would be reduced to half its value. In addition, new signals at δ 64.2 ppm (*C*H_2–OH) and δ 72.1 ppm (*C*H–OH) would be registered. No signals corresponding to the diol were observed in the ^{13}C CP/MAS NMR spectrum of the packing material. Intentional hydrolysis of the epoxide ring under the refluxing conditions with 0.1 M HCl for 2 h did not produce any significant cleavage of the epoxy ring, as deduced from NMR. Therefore, this column should not really be considered as "diol" functionalized, but rather an "epoxide" structure. The observed superior performance of this epoxy column is attributable to its stability to polar and nonpolar solvents, its incapability to form any strong hydrogen-bonded structures with proton-donor analytes, and its moderate polarity.

Influence of TAG on the quantitation of tocopherols using fluorescence detection. Figure 2 shows the relationship between different levels of triolein (10 to 400 mg) on the fluorescence response of α-T, β-T, γ-T, δ-T and 5,7-T, and α-T_3 (50 ng each). There appears to be no difference in response between the different tocopherol forms or α-T_3 to the amount of coexisting TAG. This made it possible to use any of the tocopherols as an internal standard that was not present in the sample. The similar effect of all tocopherol forms in the presence of TAG was in marked contrast to the effect of TAG after saponification in which recoveries of 90% were observed for α-T, 65% for β-T, 60% for γ-T, and 20% for δ-T at 500 mg corn oil (7). Others reported that α-T_3 was completely destroyed (16).

The recovery of all tocopherols was >95% at 25 mg/mL TAG (Fig. 2). This amount of TAG was much greater than generally observed in tissues, except adipose tissue. The neutral lipid content of most tissues was about 1 to 3%, or 0.5 to 1.5 mg/mL from a 1 g sample of tissue (since only half the hexane extract was used in the analyses). On the other hand,

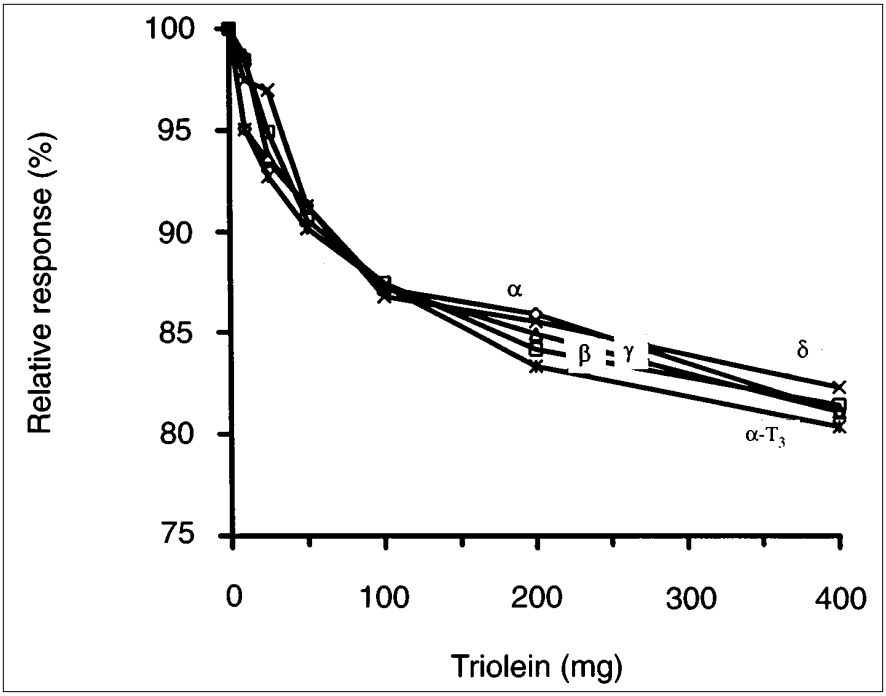


FIG. 2. The fluorescence detector response of tocopherols (T) and α-tocotrienol (α-T_3), 50 ng each, in the presence of different amounts (10 to 400 mg) of triolein. The results are means of three determinations (pooled SEM 2.33), and there were no significant differences at any concentration of triolein.

the analyses of one gram of diet, which contained 20% fat, gave 100 mg/mL TAG with an average recovery of 87% for all tocopherols, while one gram of oil gave about 400 to 500 mg/mL of TAG with an average recovery of 80% (Fig. 2). Analyses of diets and oils were corrected appropriately.

Calibration curves for the tocopherols are shown in Figure 3. The fluorescence detector response was linear over the range 10 to 100 ng of each of the vitamin E forms (all *r* values >0.99). The integrated area response for the same amount of vitamin E form was least for α-T and most for δ-T. Therefore, the following correction factors were applied for α-T (2.30), β-T (1.52), γ-T (1.32), δ-T (1.00), and α-T_3 (1.19). These results were completely contrary to those reported by Thompson and Hatina (1) that the different forms of vitamin E gave similar fluorescence detector response. Many subsequent reports have relied on these results (15,18–21,23,27,30,31), and therefore, the results were not corrected for differences in fluorescence response between the different vitamin E forms. However, two studies have recently appeared showing differences in detector responses for the various tocopherol forms using evaporative light-scattering (24) and fluorescence detection (25).

Quantitation of tocopherols in tissues. The liver and heart tocopherol values of newborn piglets fed four different experimental diets or sow milk for 4 wk are presented in Table 1. The results showed that both tissues contained mainly α-T with minor amounts of β-T, γ-T, and α-T_3, but no δ-T. These complete analyses of tissue tocopherols and tocotrienols have not been reported previously. The levels of α-T reported here were generally higher than those reported previously in pig tissues (9,10,12,14), and may indicate the success of this method to recover more of the tocopherols from tissues. Only trace amounts of vitamin E were observed in the second hexane extract of tissues which justified the use of only one hexane extraction. There were consistent diet differences, except for sow-reared piglets which showed higher liver than heart α-T (Table 1). β-T was present only in trace amounts in sow-reared piglets, but was found in piglets fed milk replacers which contained oils with β-T (see Table 2). The occurrence of β-T in either heart or liver has not been reported previously. γ-T was present at concentrations reflecting the high levels of γ-T in the dietary oils. Others have also reported the occurrence of γ-T in pig (9,12) and monkey (11) livers. In one of the studies (12), the sum of β-T and γ-T must have been reported, since a reversed-phase column was used that does not separate these two forms. We could not detect δ-T in piglet tissue, which is in agreement with another study (9). For this reason, δ-T was chosen as the internal standard for tissue tocopherol quantitation. Finally, α-T_3 was found in both liver and heart of pigs, irrespective of diet; there were generally no significant differences between diets. The occurrence of α-T_3 in pig liver was reported in one previous study in which an amino–cyano column was used (9). However, in that study, neither β-T nor γ-T was detected.

To check whether tissues contained esterified tocopherols, the residue of tissue hexane extraction was saponified and then reextracted with hexane. Less than 0.5 μg/g α-T, and no other tocopherols or α-T_3 were found in the liver, and no to-

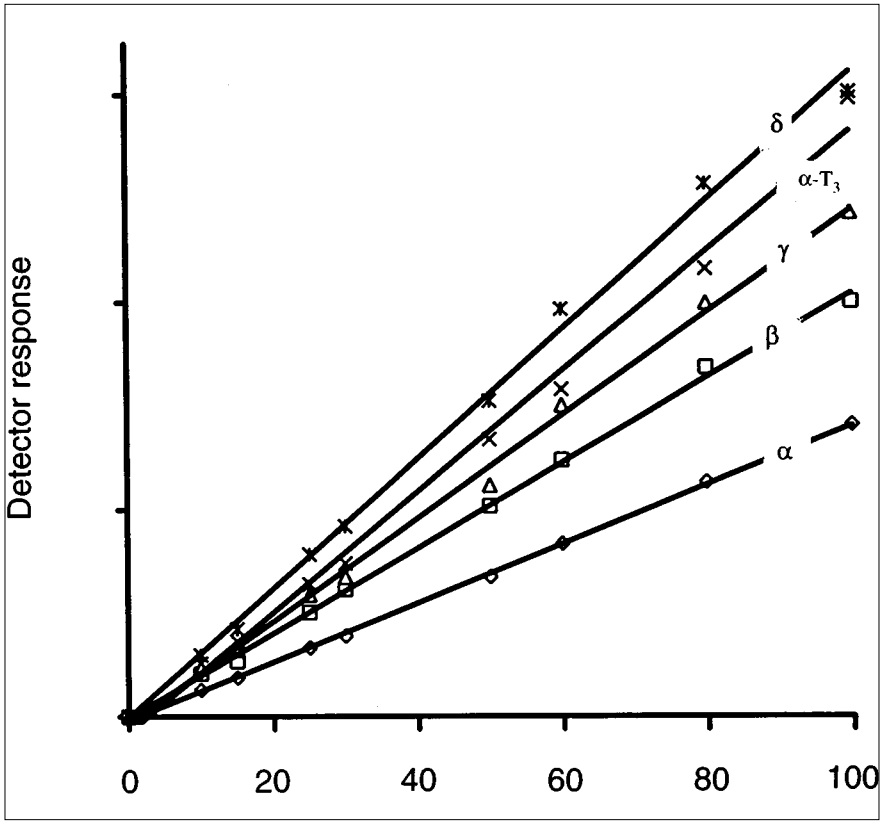


FIG. 3. Calibration curves for the fluorescence detector responses of tocopherols (T) and α-tocotrienol (α-T_3).

copherols were present in the heart after saponification. This indicated that only minor amounts of esterified tocopherol were present in liver. Incomplete extraction of the tocopherols was ruled out, because similar levels of α-T should have been found in the heart as the liver, and none was detected in the heart after saponification.

The overall error of tissue vitamin E analyses (Table 1) was large, despite randomizing diets within litter, due to variation among animals. Similar locations of the tissues were selected from the piglets to reduce variation. There was a high degree of reproducibility between repeat analyses of the same extract (data not shown). The reproducibility of duplicate determinations was also high, evident by the low error and no significant differences (Table 1). It should be noted that most of the error among determinations may be due to the fact that separate one-gram tissue samples were analyzed in this study, without prior homogenization of the total selected tissue from each piglet, and that representative subsamples were taken. The recovery of the internal standard δ-T used in the present study was 95 ± 7% (n = 60), determined by comparing the detector response of the internal standard in the sample to that of the same amount of the same tocopherol standard used externally.

Quantitation of tocopherols in diet. The analyses of the diets are shown in Table 2. Values were presented for the free tocopherols in the diet obtained by extracting with hexane and using 5,7-T as the internal standard. The latter was chosen because there was no α-T_3 present in these diets, and 5,7-T and α-T_3 had the same retention time on this column. The remaining ethanol layer was then saponified after addition of ascorbic acid and extracted to give values for α-T from the α-T succinate that was added to the diets. For the saponification step, α-T was used as an external standard to avoid differential loss during saponification of any of the other tocopherols one could have selected as an internal standard. Small amounts of the other tocopherols were also found after saponification, which suggests that other tocopherols also existed in the ester form. This modification of the method permitted the analyses of the free, naturally occurring tocopherols of diets or tissues, without differential loss during saponification, followed by a check on the amount of added tocopherol ester, usually of α-T. The recovery of the added D-α-T succinate (at 45.5 μg/g diet) in this study was 87 ± 5%.

In conclusion, a method was described to pulverize frozen tissues directly at −70°C, to eliminate the thawing and homogenization steps, and to increase the recovery of the tocopherols without the addition of any antioxidant to protect vitamin E. There was no need to remove the coeluting lipids by saponification, since they did not interfere with the quanti-

TABLE 1
Tocopherol (T) and Tocotrienol (T_3) Content of Liver and Heart from Newborn Piglets Fed Milk Replacer Diets (μg/g wet weight) or Sow Milk (for 4 wk)

	Diets			
	α-T	β-T	γ-T	α-T_3
Liver				
Sow milk	19.5[a]	0.1	0.4	1.0
Soybean oil	21.2	0.9	2.1	1.4
Canola oil	18.3	1.1	1.1	1.5
Mixture of oils[b]	10.3	1.0	1.6	1.2
HEAR oil[c]	9.9	0.7	1.0	1.3
Pooled SED[d]	3.2	0.33	0.5	0.16
Analysis of variance (mean squares)[e]				
Litters (L)	288*	0.83	2.1	0.09
Diets (D)	281**	1.33	12.1	0.31
L × D	36	0.49*	4.6**	0.15
Among piglets	60***	0.14***	0.4***	0.10**
Among determinations	1.5	0.009	0.005	0.02
Heart				
Sow milk	8.9	0.0	0.2	1.0
Soybean oil	23.3	0.3	4.0	1.2
Canola oil	16.7	0.1	1.2	1.1
Mixture of oils	15.4	0.1	2.5	1.1
HEAR oil	9.5	0.1	1.3	0.9
Pooled SED	3.5	0.05	0.5	0.33
Analysis of variance (mean squares)				
Litters (L)	270*	0.022	1.0	0.11
Diets (D)	297*	0.087***	18.3***	0.13
L × D	57	0.010	1.4	0.32
Among piglets	38***	0.011***	0.7***	0.67***
Among determinations	1.6	0.0002	0.1	0.04

[a]Each value represents the mean of two analyses/piglet and six piglets/diet.
[b]Mixture of high oleic acid sunflower oil, soybean oil, and linseed oil (55:31:14) to mimic canola oil in fatty acid composition (Ref. 33).
[c]Mixture of canola oil (0.8% 22:1n-9) and high erucic acid rapeseed (HEAR) oil (42.9%, 22:1n-9) to give a final oil containing 20% erucic acid (22:1n-9).
[d]SED, pooled maximum standard error of the difference of the two diets.
[e]Analysis of variance. Mean squares and significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

TABLE 2
Free and Esterified Tocopherol (T) Content in Milk Replacer Diets (μg/g powdered diet) of Newborn Piglets

		Diets			
	Free/esterified[a]	α-T	β-T	γ-T	δ-T
Soybean oil	Free	18.3[b]	10.8	170.8	42.4
	Esterified	40.0	2.8	29.4	4.0
Canola oil	Free	20.7	17.2	48.0	2.0
	Esterified	42.9	5.0	19.2	0.1
Mixture of oils[c]	Free	31.2	20.2	134.8	4.7
	Esterified	38.5	1.8	18.3	0.4
HEAR oil[d]	Free	41.4	23.6	119.7	3.7
	Esterified	37.9	6.3	9.9	0.1
SEM[e]	Free	1.6	1.3	10.0	1.3
	Esterified	6.6	0.1	2.0	0.1

[a]For details of how free and esterified tocopherols were measured, see the Materials and Methods section.
[b]Values are means of four determinations.
[c]Mixture of high oleic acid sunflower oil, soybean oil, and linseed oil (55:31:14) to mimic canola oil in fatty acid composition (Ref. 33).
[d]Mixture of canola oil (0.8% 22:1n-9) and high erucic acid rapeseed (HEAR) oil (42.9%, 22:1n-9) to give a final oil containing 20% erucic acid (22:1n-9).
[e]Pooled standard error of the mean.

tative analyses of the tocopherol forms, except when tocopherol esters were measured that were added to the diet. The fluorescence response was different for the various tocopherol forms, affected slightly by the presence of large amounts of TAG, but the latter response was similar for all tocopherol forms. For tissue analyses, the response was >95%, and for oils it was about 80%. The normal-phase diol column for HPLC gave reproducible and consistent separations of all the tocopherol and tocotrienol forms, without the disadvantages of nonreproducibility when silica columns were used, or the lack of separation with reversed-phase columns.

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Analysis of Lipoxygenase-Derived Fatty Acid Hydroperoxides by Electrospray Ionization Tandem Mass Spectrometry

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ABSTRACT: A rapid method is described for the identification of fatty acid hydroperoxides by electrospray ionization–tandem mass spectrometry coupled to liquid chromatography without any derivatization required prior to analysis. Localization of fatty acid hydroperoxides in complex mixtures was achieved by monitoring the loss of hydrogen peroxide using constant neutral loss scanning. In the presence of 5 mM NH_4OAc in methanol–water, adduct ions $[M + NH_4]^+$ were formed almost exclusively, directly revealing the molecular mass of the thermolabile hydroperoxides. In addition, low energy collision-induced dissociation of precursor ions $[M + NH_4]^+$ led to characteristic product ions from both the 9- and 13-regioisomers. Thus, electrospray ionization–tandem mass spectrometry provides a straightforward approach for the study of the regioselectivity of lipoxygenase catalysis without any derivatization step required prior to analysis.

Lipids 32, 331–336 (1997).

Fatty acid hydroperoxides are labile key intermediates in plant and mammalian lipid metabolism, acting as progenitors of a variety of lipid-derived mediators such as prostaglandins and leukotrienes (1,2). They are formed by lipoxygenase (LOX) action on polyunsaturated fatty acids containing a 1,4-*Z,Z*-pentadiene moiety such as linoleic and linolenic acid. LOX from different sources not only show distinct enantioselectivity but also exhibit a characteristic positional specificity (3): whereas soybean lipoxygenase-1 selectively produces 13-*S*-hydroperoxides, LOX isoenzymes 2 and 3 from the same source generate a racemic mixture of both 9- and 13- isomers (3). In contrast, tomato LOX almost exclusively yields the 9-*S*-hydroperoxides (4).

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Abbreviations: CID, collision-induced dissociation; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; HPOD, hydroperoxyoctadecadienoic acid; 13-*S*-HPOD, 13-*S*-hydroperoxy-9-*Z*,11-*E*-octadecadienoic acid; 9-*S*-HPOD, 9-*S*-hydroperoxy-10-E,12-*Z*-octadecadienoic acid; HPOT, hydroperoxyoctadecatrienoic acid; 9-*S*-HPOT, 9-*S*-hydroperoxy-10-*E*,12-*Z*,15-*Z*-octadecatrienoic acid; 13-*S*-HPOT, 13-*S*-hydroperoxy-9-*Z*,11-*E*,15-*Z*-octadecatrienoic acid; LC, liquid chromatography; LOX, lipoxygenase; MS/MS, tandem mass spectrometry; THF, tetrahydrofuran; UV, ultraviolet.

The conventional analysis of the thermolabile regioisomeric fatty acid hydroperoxides by gas chromatography–mass spectrometry requires a tedious derivatization procedure including reduction of the hydroperoxide moiety followed by methylation of the carboxy function and silylation of the hydroperoxide-derived hydroxyl group (5). Nonetheless, specific detection of 9- and 13-regioisomers has proved to be difficult, since the resulting trimethylsilyl-derivatives exhibit very similar chromatographic and spectroscopic properties (6). Thus, hydrogenation of the carbon-carbon double bond has to be included as an additional derivatization step prior to gas chromatography–mass spectrometry analysis.

Recently, development of electrospray ionization (ESI) has pioneered the coupling of liquid chromatography (LC) with mass spectrometry (7). In particular, the efficient on-line characterization of polar and labile intermediates by tandem mass spectrometry (MS/MS) in combination with electrospray ionization (ESI) opens further dimensions in the field of bio-organic analysis. By LC–ESI–MS/MS, both molecular mass and substructure-specific data are available without the need of derivatization and laborious sample clean-up.

Spurred by recent reports on the analysis of lipid-derived mediators such as carboxylate anions, applying ESI in the negative mode (8,9), we developed a strategy based on LC–ESI–MS/MS for the detection of fatty acid hydroperoxides in complex samples followed by identification of the corresponding regioisomers.

MATERIALS AND METHODS

Chemicals and materials. Linoleic acid (99% pure) was obtained from Fluka (Neu-Ulm, Germany). Linolenic acid (99% pure), ammonium acetate, and soybean LOX (EC 1.13.11.12) (type I-S, L-8383) were from Sigma (Steinheim, Germany). High-performance liquid chromatography (HPLC)-grade solvents, water, and methanol were from Merck (Darmstadt, Germany); tetrahydrofuran (THF) from Roth (Karlsruhe, Germany); and acetic acid from Baker Chemicals (Deventer, The Netherlands). Tomatoes were purchased at the local market. All other chemicals were of analytical grade quality; solvents were redistilled before use.

Isolation of LOX. Tomato LOX was prepared using a modified procedure described by Bonnet and Crouzet (10). Five hundred grams of tomato fruit (*Lycopersicon esculentum*) were washed and cooled at 4°C, cut into slices, and blended in a mixer with 500 mL 0.5 M Tris-HCl buffer (pH 8.0) containing 1% (wt/vol) ascorbic acid, 1% (wt/vol) EDTA, and 1 mM phenylmethylsulfonylfluoride for 3 min at 4°C. The homogenate was filtered through two layers of cheesecloth. To the filtrate, 2% (vol/vol) 1 M $CaCl_2$ was added and 2 h later the suspension was centrifuged at 15,000 × *g* for 20 min. Protein was precipitated by adding solid ammonium sulfate up to 60% saturation to the supernatant. The precipitate was collected by centrifugation at 15,000 × *g* for 20 min, dissolved in 5 mL of 0.1 M phosphate buffer (pH 6.5), and dialyzed overnight against the same buffer. This solution (0.7 U LOX/mL) was used as the source of LOX.

Soybean LOX-2 was purified from soybean seeds as previously described (11) and freeze-dried after addition of 0.1 N sodium chloride. This preparation (12 U LOX/mg) was used as the source of LOX-2.

Preparation of fatty acid hydroperoxides. 13-*S*-hydroperoxy-9-*Z*,11-*E*-octa-decadienoic acid (13-*S*-HPOD) and 13-*S*-hydroperoxy-9-*Z*,11-*E*,15-*Z*-octadecatrienoic acid (13-*S*-HPOT) were prepared using soybean LOX (Sigma, L-8383). Typically, 5 mg fatty acid was diluted in 10 mL of 0.1 M borate buffer (pH 9.0) in a conical flask. The solution was mixed by magnetic stirring and flushed with oxygen while the temperature was maintained at 0°C. The reaction was initiated by addition of 1 mg soybean LOX; progress of the enzymatic conversion was monitored photometrically at 234 nm. When the reaction was almost completed, the pH was adjusted to pH 3 with 1 N HCl. The solution was saturated with sodium chloride, and the mixture was extracted three times with 5 mL of diethyl ether each. The combined extracts were washed with water, dried over sodium sulfate, and evaporated *in vacuo*. The residue was dissolved in methanol and further purified by reversed-phase HPLC.

9-*S*-hydroperoxy-10-*E*,12-*Z*-octadecadienoic acid (9-*S*-HPOD) and 9-*S*-hydroperoxy-10-*E*,12-*Z*,15-*Z*-octadecatrienoic acid (9-*S*-HPOT) were prepared using tomato LOX. Five mg fatty acid was dissolved in 10 mL of 0.1 M phosphate buffer (pH 6.5) and maintained at 0°C. The solution was flushed with oxygen and stirred magnetically. The reaction was started adding 1 mL of freshly prepared tomato LOX and allowed to proceed for about 5 h. The solution was adjusted to pH 3.0 with 1 N HCl and extracted as described above.

Conversion of linoleic acid with soybean LOX-2 was carried out as described for the preparation of 13-*S*-isomers using 0.1 M phosphate buffer (pH 6.5) and limiting reaction time to 2 h.

For estimation of regioselectivity hydroperoxides derived from LOX-2 oxidation were quantified using product ions *m/z* 195 (13-HPOD) and *m/z* 155 (9-HPOD):

$$\frac{\text{9-HPOD}_{\text{sample}}}{\text{13-HPOD}_{\text{sample}}} = \left[\frac{\text{intensity } m/z\ 155}{\text{intensity } m/z\ 195}\right]_{\text{sample}} \times \left[\frac{\%\ m/z\ 195}{\%\ m/z\ 155}\right]_{\text{reference}} \quad [1]$$

A complex biological sample for detection of fatty acid hydroperoxides was prepared using a crude enzyme extract from soybean. One gram homogenized and defatted soybean seeds was extracted for 1 h with 10 mL of 0.2 M sodium acetate buffer pH 4.5 containing 5 mM EDTA. After centrifugation, the supernatant was used to oxidize 10 mg of linoleic acid in 20 mL of 0.1 M phosphate buffer pH 6.5 at ambient temperature. Work-up was as described above.

Chromatographic purification of fatty acid hydroperoxides as reference compounds. Fatty acid hydroperoxides were further purified by reversed-phase HPLC (12). The isomeric LOX products were analyzed on a Spherisorb ODS-2 column (5 µm, 4.6 × 250 mm) (Knauer, Berlin, Germany) using an ultraviolet (UV) detector (variable wavelength detector, Knauer) set to 234 nm for detection of conjugated dienes. Isocratic elution (HPLC pump 64, Knauer) was performed with THF/MeOH/water/acetic acid (18:48:33.9:0.1, by vol) at a flow rate of 0.8 mL/min. Semipreparative purification of hydroperoxides required multiple injections and collection of the corresponding peaks. The purified hydroperoxides were evaporated *in vacuo* and dissolved in the starting buffer of HPLC analysis to a final concentration of 0.026 mM. Configuration of double bonds in 9- and 13-HPOD was assigned by HPLC with UV detection according to standard procedure (13).

Mass spectrometric analysis of fatty acid hydroperoxides. For LC–ESI–MS/MS, chromatographic separation was performed on Eurospher 100 C-18 column (5 µm, 2 × 100 mm; Knauer) with a binary gradient delivered by an Applied Biosystems 140B pump (Foster City, CA). Solvent A was THF/MeOH/water/acetic acid (25:30:44.9:0.1, by vol), solvent B was MeOH/water (9:1, vol/vol). Both solvents A and B contained 5 mM NH_4OAc. HPLC was programmed as follows: pressurizing 1 min with 50% solvent A at 1 mL/min, target time 1 min at 0.2 mL/min, equilibration time 5 min at 90% solvent A, gradient elution: 0 min to 5 min 90% A isocratically, 5 to 10 min 90% A to 50% A and 10 min to 15 min isocratically at 50% A with a flow rate of 0.2 mL/min. Injection volume was 1 µL for reference compounds (30 pmol on column) and 5 µL for biological samples (50 pmol on column), respectively. HPLC–MS analysis of all reference compounds and biological samples was performed on a Finnigan TSQ 7000 tandem mass spectrometer (Finnigan MAT, Bremen, Germany) which was tuned with MRFA-(Met-Arg-Phe-Ala)-myoglobin solution. For pneumatically assisted ESI the spray capillary voltage was set to 4 kV and the temperature of the heated inlet capillary serving simultaneously as repeller electrode (20 V) was 180°C. Nitrogen served both as sheath (70 psi) and auxiliary gas (10 L/min). Positive and negative ions were detected scanning from 250 to 400 u with a total scan duration of 1.0 s for a single full spectrum. MS/MS experiments were performed at a collision gas pressure of 1.8 mTorr Argon scanning a mass range from 20 to 350 u with a total scan duration of 3.0 s for a single full spectrum. All spectra presented reflect the average of five or more full scans which were corrected for background.

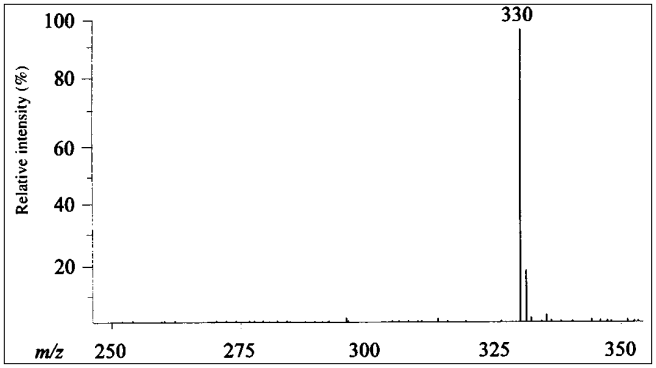


FIG. 1. Positive ion electrospray ionization–mass spectroscopy spectrum of 13-*S*-hydroperoxy-9-*Z*,11-*E*-octadienoic acid.

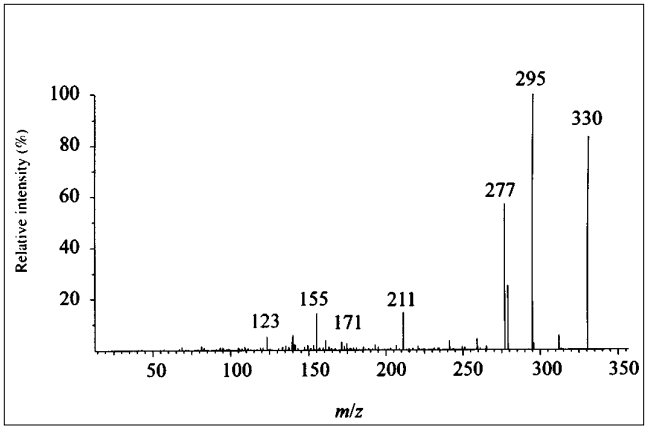


FIG. 2. Product ion spectrum of 9-*S*-hydroperoxy-10-*E*,12-*Z*-octadecadienoic acid), precursor ion *m/z* 330 (positive mode, –7 eV, 1.8 mTorr argon as collision gas).

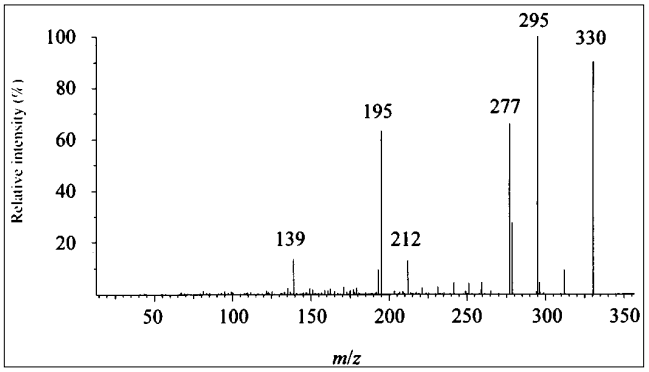


FIG. 3. Product ion spectrum of 13-*S*-hydroperoxy-9-*Z*-11-*E*-octadienoic acid, precursor ion *m/z* 330 (positive mode, –7 eV, 1.8 mTorr argon as collision gas).

RESULTS AND DISCUSSION

Molecular mass determination of fatty acid hydroperoxides by LC–ESI–MS. LC–ESI–MS analysis in methanol–water–ammonium acetate of pure 9- and 13-*S*-HPOD and 9- and 13-*S*-HPOT revealed prominent adduct ions $[M + NH_4]^+$ with *m/z* 330 for 9/13-*S*-HPOD (Fig. 1) and *m/z* 328 for 9/13-*S*-HPOT. In contrast, acetonitrile–water–trifluoroacetic acid as solvent failed to yield abundant protonated molecular ions.

By setting the temperature of the heated inlet capillary to 180°C, only negligible intensities (less than 5%) of dehydrated ions *m/z* 312 (hydroperoxyoctadecadienoic acid, HPOD, Fig. 1) and *m/z* 310 (hydroperoxyoctadecatrienoic acid, HPOT) were detected. Further reduction of the heated capillary temperature did not result in higher intensities of adduct ions $[M + NH_4]^+$ because of problems with incomplete desolvation and formation of cluster ions $[M + (MeOH)_n + NH_4]^+$ (n = 1–3). The solvent system MeOH–water containing 5 mM NH_4OAc also allowed detection of anions without change of eluent; thus, deprotonated molecular ions $[M - H]^-$ *m/z* 311 and *m/z* 309 were generated from HPOD and HPOT, respectively. Again, loss of water was negligible (about 5%).

MS/MS analysis of fatty acid hydroperoxides. Product ion spectra obtained by low-energy collision-induced dissociation (CID) (7 eV) of $[M + NH_4]^+$ precursor ions derived from fatty acid hydroperoxides revealed characteristic fragment patterns (Figs. 2 and 3). The most abundant product ions in both 9-/13-HPOD and HPOT resulted from the consecutive loss of water

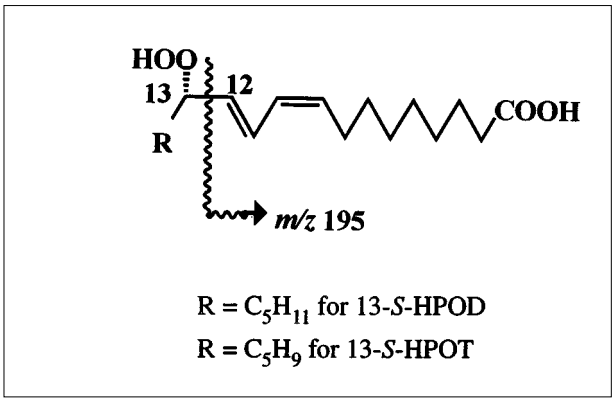
TABLE 1
Product Ions (*m/z*) Obtained by CID of Fatty Acid Oxidation Products[a,b]

	9-*S*-HPOD (Fig. 2)	12-*S*-HPOD (Fig. 3)	9-*S*-HPOT	13-*S*-HPOT	9-/13-KOD
$[M + NH_4]^+$	330	330	328	328	312
$[M + H]^+$	n.d.	n.d.	n.d.	n.d.	295
$[M - H_2O + NH_4]^+$	312	312	310	310	n.d.
$[M - H_2O + H]^+$	295	295	293	293	277
$[M - H_2O_2 + H]^+$	279	279	277	277	n.d.
$[M - 2 \times H_2O + H]^+$	277	277	275	275	n.d.
$[C_{12}H_{19}O_2]^+$	n.d.	195	n.d.	195	n.d.
$[C_9H_{17}O_4 - H_2O]^+$	171	n.d.	171	n.d.	n.d.
$[C_9H_{17}O_4 - H_2O_2]^+$	155	n.d.	155	n.d.	n.d.
	211	n.d.	211	n.d.	n.d.
	n.d.	212	n.d.	212	n.d.

[a]Conditions: precursor ions: $[M + NH_4]^+$; collision gas: 1.8 mTorr Ar; collision energy: –7eV.
[b]Abbreviations: CID, collision-induced dissociation; 9-*S*-HPOD, 9-*S*-hydroperoxy-10-*E*,12-*Z*-octadecadienoic acid; 13-*S*-HPOD, 13-*S*-hydroperoxy-9-*Z*,11-*E*-octadecadienoic acid; 9-*S*-HPOT, 9-*S*-hydroperoxy-10-*E*,12-*Z*,15-*Z*-octadecatrienoic acid; 13-*S*-hydroperoxy-9-*Z*,11-*E*,15-*Z*-octadecatrienoic acid; KOD, keto-octadecadienoic acid; n.d., not detected.

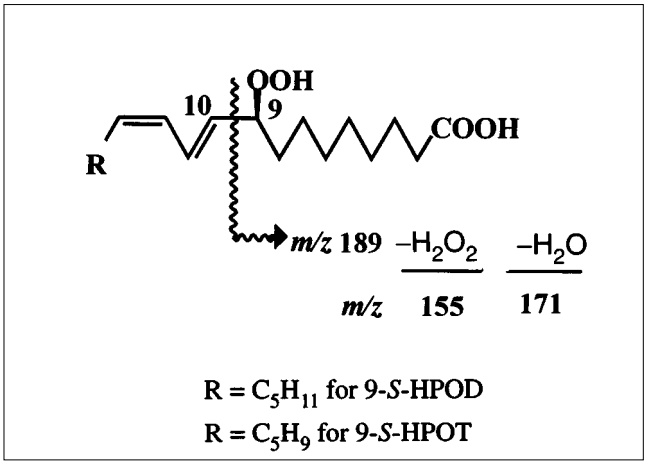
(Table 1). Product ions indicative for the hydroperoxy moiety were formed by loss of hydrogen peroxide which resulted in *m/z* 279 from 9/13-HPOD and *m/z* 277 from 9/13-HPOT. This fragmentation pathway was confirmed by corresponding neutral loss experiments, which clearly revealed the elimination of either H_2O_2 (–34 u) or [H_2O_2 + NH_3] (–51 u) from $[M + H]^+$ and $[M + NH_4]^+$ precursor ions, respectively. Control experiments with 13-*S*-HOD exclusively showed elimination of water (–18 u). Thus the loss of 34 u or 51 u was indicative for the hydroperoxy moiety and allowed detection of hydroperoxides in complex matrices by constant neutral loss scanning.

Product ions *m/z* 195 and *m/z* 212 were characteristic for 13-regioisomers (Fig. 3). The prominent ion *m/z* 195 $[C_{12}H_{19}O_2]^+$ apparently was formed by cleavage of the C_{12}–C_{13} bond (see Scheme 1) as it occurred in both 13-*S*-HPOD and 13-*S*-HPOT. A similar fragmentation has been observed while studying 13-HPOD applying negative ESI followed by CID of the molecular anion (9).



SCHEME 1

In case of 9-regioisomers (Fig. 2), characteristic product ions *m/z* 171 ($[C_9H_{17}O_4 - H_2O]^+$) and *m/z* 155 ($[C_9H_{17}O_4 - H_2O_2]^+$) resulted from the fission of the corresponding C_9–C_{10} bond and subsequent elimination of water or H_2O_2, respectively (see Scheme 2):



SCHEME 2

In addition, fragment *m/z* 211 was characteristic for 9-regioisomers as *m/z* 212 was for 13-regioisomers, but the identity of these ions remains to be established.

As can be seen from the characteristic product ions of both 9- and 13-regioisomers, a preferred and diagnostic fragmentation of unsaturated hydroperoxides was observed which is based on an α-cleavage between the carbon bearing the hydroperoxy moiety and the carbon of the adjacent double bond. Thus, not only did MS/MS allow localization of fatty acid hydroperoxides by monitoring the loss of H_2O_2, but also product ion spectra of unsaturated hydroperoxides enabled assignment of the corresponding regioisomers without any derivatization step required prior to analysis.

LC–ESI–MS/MS analysis of linoleic acid oxidation products in complex samples. Soybean LOX-2 initiated oxidation of linoleic acid is known to produce a mixture of 9- and 13-HPOD (3). In addition, the corresponding keto and hydroxy fatty acids as well as by-products have been observed (2). Thus, we incubated linoleic acid with soybean LOX-2 and analyzed the reaction products directly by means of LC–ESI–MS/MS. The only clean-up step required prior to analysis was separation of fatty acid-derived products from protein in the incubation mixture. Subsequently, the crude extract was directly analyzed by monitoring product ions of precursor ion *m/z* 330 ($[M + NH_4]^+$). Under the chromatographic conditions selected, 9- and 13-HPOD were not separated in the total ion chromatogram (Fig. 4, lower trace) as well as in the UV trace (235 nm, data not shown). Nevertheless, the reconstructed ion chromatogram for *m/z* 195 and *m/z* 155 clearly demonstrated the presence of both 13- and 9-HPOD. Thus, even fatty acid hydroperoxides which appeared by UV-detection to coelute could be identified unequivocally by LC–ESI–MS/MS.

Interpretation of the chromatogram in Figure 4 revealed a ratio of 9- to 13-HPOD of 1 to 1.3 according to the intensities of *m/z* 155 and *m/z* 195 in the spectra. This ratio was in good agreement with results obtained by the standard procedure after extraction, reduction, and methylation followed by normal phase HPLC (data not shown).

As outlined before, product ion spectra of all fatty acid hydroperoxides under investigation showed a characteristic loss of hydrogen peroxide. As a demonstration of the poten-

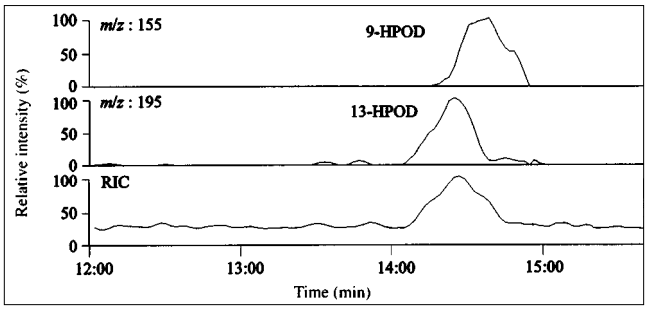


FIG. 4. Liquid chromatography–tandem mass spectrometry analysis of soybean lipoxygenase-2 oxidation of linoleic acid. Product ions of *m/z* 330 (positive mode, –7 eV, 1.8 mTorr argon as collision gas). Abbreviations: 9-HPOD, 9-hydroperoxyoctadecadienoic acid; 13-HPOD, 13-hydroperoxyoctadecadienoic acid; RIC, reconstructed ion chromatogram..

tial of the constant neutral loss scanning as an efficient screening technique for detection of fatty acid hydroperoxides, a reaction mixture obtained by incubation of linoleic acid with a crude enzyme preparation from soybeans was analyzed.

Prior to MS/MS experiments, the resolution of both quadrupole mass filters was adjusted to 0.6 u (full peak width at half maximum height) in order to exclude interference between neutral loss of 34 u (H_2O_2) and 35 u ($H_2O + NH_3$). Monitoring the loss of 34 u or 51 u ($H_2O_2 + NH_3$), respectively, led to detection of 9- and 13-HPOD together with monohydroperoxides with *E,E*-configured double bonds (Fig. 5, trace C).

In addition, one putative hydroperoxide showing a prominent precursor ion *m/z* 346 $[M + NH_4]^+$ was localized among the more polar reaction products (Fig. 5, upper trace). By ESI in the negative mode, a deprotonated molecular ion *m/z* 327 was observed, establishing a molecular mass of 328 u for the polar hydroperoxide.

Additional LC–MS/MS experiments revealed the presence of 9- and 13-keto-octadecadienoic acids (Table 1) and, to a smaller extent, the presence of hydroxyoctadecadienoic acids, which were identified by their molecular mass and UV spectra (Fig. 5, trace B). Again, LC–MS/MS proved to be more reliable than UV detection because structures of even coeluting compounds were assigned successfully.

In summary, we detected adduct ions $[M + NH_4]^+$of fatty acid hydroperoxides formed by ESI. MS/MS experiments allowed structure-specific detection of these metabolites in complex samples. After low-energy CID of $[M + NH_4]^+$ precursor ions, 9- and 13-regioisomeric fatty acid hydroperoxides were identified by their characteristic product ion spectra. Thus, LC–ESI–MS/MS proved to be more reliable than UV detection, because structures of even coeluting compounds were successfully assigned. This method opens a versatile analytical approach for the structure-specific determination of labile lipid mediators in biological samples.

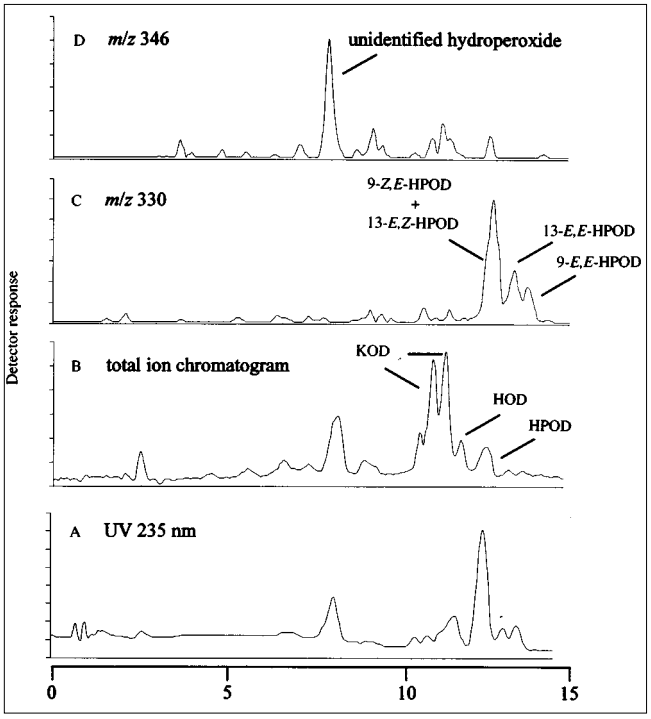


FIG. 5. Liquid chromatography–mass spectroscopy, liquid chromatography–tandem mass spectroscopy, and ultraviolet analysis of linoleic acid oxidized by crude soybean extract. (A) ultraviolet, 235 nm. (B) Liquid chromatography–mass spectroscopy reconstructed total ion chromatogram, positive mode, scan range *m/z* 250–400. (C) and (D) Liquid chromatography–tandem mass spectroscopy analysis monitoring neutral loss of H_2O_2 (positive mode, –7 eV, 1.8 mTorr argon as collision gas): (C) reconstructed ion chromatogram for precursor ion *m/z* 330; (D) reconstructed ion chromatogram for precursor ion *m/z* 346; HPOD, hydroperoxyoctadecadienoic acid; Abbreviations: HOD, hydroxyoctadecadienoic acid; KOD, keto-octadecadienoic acid; UV, ultraviolet.

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Stable-Isotope Method for Determining the Gastrointestinal Handling of [1-^{13}C]Palmitic Acid

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ABSTRACT: The ^{13}C enrichment in individual fatty acids extracted from human feces following the oral administration of [1-^{13}C]palmitic acid has been determined using a novel approach based upon gas chromatography–isotope ratio mass spectrometry. The method was established and tested for precision and repeatability. Analytical precision was determined from 10 repeated injections of a sample containing 16:0 and 18:0 with levels of δ^{13}C abundance measured at –34.01 ± 0.60 and –23.62 ± 0.95 delta per mil (parts per thousand) (‰), respectively (mean ± SD). For the repeatability study, measurement of enrichment of the same mixture of unlabeled fatty acid methyl ester (FAME) standards (13:0, 14:0, 16:0, and 18:0) was found to have standard deviations (0.45, 0.56, 1.46 and 1.54‰, respectively). When labeled [1-^{13}C]palmitic acid was serially diluted with naturally enriched palmitic acid, a linear relationship was obtained to a dilution of 10% enriched compound (530‰). FAME were prepared from two fecal samples from a normal healthy adult; the first, a baseline specimen, containing no added label and the second, followed a single oral dose of [1-^{13}C]palmitic acid and was enriched. Enrichment in ^{13}C was confined to the solvent-soluble fraction following lipid extraction, and was only identified with prior acidification. The enrichments were measured in triplicate, baseline sample –32.66 ± 0.5‰, enriched sample +268.61 ± 8.0‰. Enrichment was restricted to the labeled species consumed, 16:0. The methodology described here allows for the separation of compounds prior to the determination of enrichment and can be utilized to contribute to a more complete description of the gastrointestinal handling of labeled substrates than previously obtained.

Lipids 32, 337–340 (1997).

There are few good noninvasive approaches to characterize how dietary lipid is handled by the gastrointestinal tract in children and adults. The most frequently used approach has been to conduct balance studies in which either the total lipid content of feces (1) or the concentrations of individual fatty acids in feces are determined (2,3). By using these approaches, it is not possible to determine with confidence whether the lipid measured in feces is derived directly from the diet, or carries a contribution from endogenous sources, such as biliary and other secretions, desquamated cells, or even the products of bacterial metabolism. As an alternative, fatty acids labeled with ^{14}C and ^{13}C have been used to determine the extent of digestion and absorption of dietary lipid (4). For example, Jones *et al.* (5) were able to assess the extent to which [1-^{13}C] stearic, oleic, and linoleic acids were absorbed from the rate of excretion of ^{13}C-label in feces using isotope ratio mass spectrometry (IRMS). They found that the apparent absorption of individual fatty acids, orally administered as the free acid, increased with degree of unsaturation. The methods used in these studies were complex and involved extensive preparative procedures.

We have previously determined the fate of label from orally administered [1-^{13}C]palmitic acid in healthy young women, by determining the proportion of label excreted in feces. In this study, the total ^{13}C enrichment in feces was measured using automated continuous flow (CF)–IRMS (CF–IRMS) (6). It was not possible to determine the nature of the species bearing the ^{13}C label, and the assumption was made that the label in feces was in the same form as that ingested. The introduction of on-line separation of fatty acids by gas chromatography–IRMS (GC–IRMS) makes it possible to determine the level of enrichment of specific fatty acids in biological specimens (7). Recently, Binnert and coworkers (8) have reported the application of GC–IRMS to determine the movement of label from ingested triacylglycerol to circulating lipids in blood.

In the present report, we describe a method for the on-line separation and determination of enrichment of fatty acids extracted from fecal samples. The precision and repeatability of the technique to measure the enrichment in fatty acid methyl ester (FAME) standards and also the ^{13}C abundance of long-chain fatty acids present in feces were determined. The results show that this relatively simple methodology can be used on a routine basis to characterize the nature of the compounds bearing the label within feces.

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Abbreviations: APE, atom percentage excess; CF–IRMS, continuous flow–isotope ratio mass spectrometry; FAME, fatty acid methyl esters; FID, flame–ionization detector; GC, gas chromatography; GC–IRMS, gas chromatography–isotope ratio mass spectrometry; GC–MS gas chromatography–mass spectrometry; IRMS, isotope ratio mass spectrometry; MPE, mole percentage excess; PDB, pee dee belemnite; ‰, delta per mil (parts per thousand).

EXPERIMENTAL PROCEDURES

Chemicals. Chemicals were obtained from Sigma Chemical Co. Ltd. (Poole, United Kingdom) unless otherwise stated. All solvents were of Analar grade or high-performance liquid chromatography (HPLC) grade with hexane 99% pure as determined by capillary GC.

Measurement of ^{13}C-enrichment. For the measurement of total ^{13}C enrichment, 2 mg samples were weighed into tin capsules (Elemental Microanalysis, Okehampton, United Kingdom) and analyzed by CF–IRMS (ANCA system; Europa Scientific Ltd., Crewe, United Kingdom) using methods previously described (6).

The determination of enrichment in FAME was performed on a gas chromatograph (Model 5890, Hewlett-Packard, Palo Alto, CA) interfaced to an IRMS (Orchid GC interface and ANCA-*NT* IRMS; Europa Scientific Ltd. Fatty acids were separated by GC as methyl esters on a BPX-70 column (33 mm i.d., 50 m; SGE Ltd, Milton Keynes, United Kingdom). Helium was used as the carrier gas at a column head pressure of 120 kPa. The injector port temperature was set at 270°C, and the detector set at 295°C. Samples were injected split mode and then detected by flame-ionization detector (FID). FAME were identified by comparison with known standards. For isotopic mass spectrometric analysis following GC separation, the sample was diverted to a combustion tube containing platinized copper oxide powder (Elemental Microanalysis) maintained at 800°C. Water was removed by the use of a hygroscopic ion exchange membrane (Perma Pure Inc., Toms River, NY) in a continuous drying treatment. This process resulted in discrete samples of CO_2 flowing directly into the source of the IRMS. The mass spectrometer was equipped with triple collectors for the simultaneous recording of CO_2^+ ions. The instrument was calibrated to measure the major CO_2^+ ions: m/z = 44[$^{12}C^{16}O^{16}O$], 45[$^{13}C^{16}O^{16}O$ or $^{12}C^{17}O^{16}O$], and 46[$^{12}C^{18}O^{16}O$]. Data processing and enrichment measurements were determined using the software provided by the manufacturer (VANCA-GC; Europa Scientific Ltd.). Enrichment of FAME standards were calculated automatically against undecanoic acid (11:0) with a known enrichment value of –27.45‰. Enrichments of FAME obtained from fecal samples were calculated against tricosanoic acid (23:0; –32.45‰) added prior to the injection of the sample. All samples were injected in amounts calculated to give peak areas of comparable beam strengths to those used with the standards.

Validation studies. With all FAME standards, the column temperature was held at 160°C for 2 min and then increased to 280°C at 4°C/min. The precision of the system was calculated from ten consecutive 1 μL injections of palmitic acid (16:0), and stearic acid (18:0), from a stock solution of 2 mg/mL total FAME dissolved in hexane. For the repeatability study, the enrichment of a mixture of FAME standards—tridecanoic acid (13:0), myristic acid (14:0), palmitic acid (16:0), and stearic acid (18:0)—was measured on 35 occasions over a 4-mon period. The FAME standards were obtained from Europa Scientific Ltd. and dissolved in hexane at a concentration of 2 mg/mL and stored at 4°C. All measurements were separated by at least 24 h with the mass spectrometer tuned for the optimal signal prior to each occasion.

A calibration curve was obtained by measuring enrichment following the addition of increasing proportions, from 0 to 10%, of [1-^{13}C]palmitic acid (99 APE; Mass Trace Technologies Inc., Somerville, MA) to natural palmitic acid, 2 mg/mL for each injection.

Efficiency of label extraction. Fecal samples, known to be enriched, were obtained from a previous study. They were collected from a normal healthy adult on the third day following a single oral dose of [1-^{13}C]palmitic acid (10 mg/kg body weight). The feces had been freeze-dried and aliquots of dry powder, 100 mg, were taken for lipid extraction. Lipids were extracted from feces in two ways: the method of Folch *et al.* (9) was used with and without prior acidification. Samples were acidified to pH <3 by the addition of 6 M HCl. The solvent wash as well as the remaining residue following solvent extraction for both the acidified and nonacidified samples was then freeze-dried (Genevac, Ipswich, United Kingdom). All fractions were analyzed for total enrichment by CF–IRMS as described above.

Determining the nature of label within stool. The nature of the label within stool was determined by GC–IRMS following the addition of 0.5 mg of heptadecanoic acid (17:0) to 100 mg dry fecal matter as an internal standard. Lipids were saponified with KOH, methylated in 1% H_2SO_4 in methanol overnight at 50°C with FAME taken up in hexane for GC–IRMS analysis. For GC analysis, the column temperature was held at 160°C for 2 min, increased to 170°C at 4°C/min and held for 15 min. This was followed by a ramp to 280°C at 10°C/min and held at this temperature for 3.5 min.

Notation/calculations. The standard notation for the expression of enrichment results is ‰, defined in Equation 1 as:

$$\delta^{13}C(‰) = \frac{\left(^{13}C/^{12}C_{SAMPLE}\right) - \left(^{13}C/^{12}C_{PDB}\right)}{\left(^{13}C/^{12}C_{PDB}\right)} \times 1000 \qquad [1]$$

where PDB (Pee Dee Belemnite) refers to the ^{13}C abundance of an international standard. For a compound with a lower natural abundance than PDB, the value for enrichment will be negative; the lower the natural abundance of a compound the more negative the value. For carbon isotopes, a 1% excess in fractional ^{13}C content relative to natural abundance corresponds to approximately 1000‰.

Results for the standard curve were expressed as moles percentage excess (MPE) as described by Binnert *et al.* (8) to enable comparison between different GC–IRMS systems. Values were determined by converting the atom percentage excess (APE), calculated by subtracting the basal ^{13}C abundance of palmitic acid from the ^{13}C enrichment of the samples containing the label and multiplying by 17 (the number of carbon atoms in the derivatized molecule).

All results are expressed as mean ± SD; associations between variables were tested by the Pearson product moment correlation coefficient (r).

RESULTS

Validation studies. To determine precision, the enrichment in two fatty acids was measured in 10 consecutive injections and was −34.01 ± 0.60‰ for 16:0 and −23.62 ± 0.95‰ for 18:0. For repeatability, measurement of ^{13}C enrichment of the same mixture of unlabeled FAME standards (13:0, 14:0, 16:0, and 18:0) on 35 different occasions were found to have standard deviations of (0.45, 0.56, 1.46 and 1.54‰, respectively).

The standard curve showed a linear relationship between measured enrichment of palmitic acid with increasing proportion of labeled fatty acid added to unlabeled fatty acid. There was a progressive increase in the measured enrichment as labeled palmitic acid was added to unlabeled palmitic acid, from −31.23‰ for baseline enrichment of unlabeled fatty acid to 530‰ when 10% of the total palmitic acid was present in the labeled form. The equation of the line was y = 53.80x − 31.57; r = 0 .9999

Extraction of ^{13}C label from feces. Acidification of the sample, prior to lipid extraction, resulted in the recovery of label in the solvent extract of feces, with no label identified in the residue. When the acidification step was omitted, the recovery of label in the solvent extract was significantly reduced, with a substantial part of the label being measured in the residue which remained after solvent extraction (Table 1).

The nature of stool lipid. Figure 1 shows a GC–IRMS chromatogram of fatty acids extracted from a sample of human feces, for the *m/z* = 44 signal. Peak 2 represents 17:0, an internal standard added prior to lipid extraction. Peak 6 represents 23:0, a standard of known enrichment against which the enrichment in all other peaks could be compared. The main peaks identified correspond to the following fatty acids: 16:0 (peak 1), 18:0 (peak 3), 18:1 (peak 4), and 18:2 (peak 5). Table 2 shows the enrichment of the major FAME in the two fecal samples. The results show a large rise in enrichment in 16:0, the labeled species consumed by the subject, with virtually no change in enrichment in any other detected FAME.

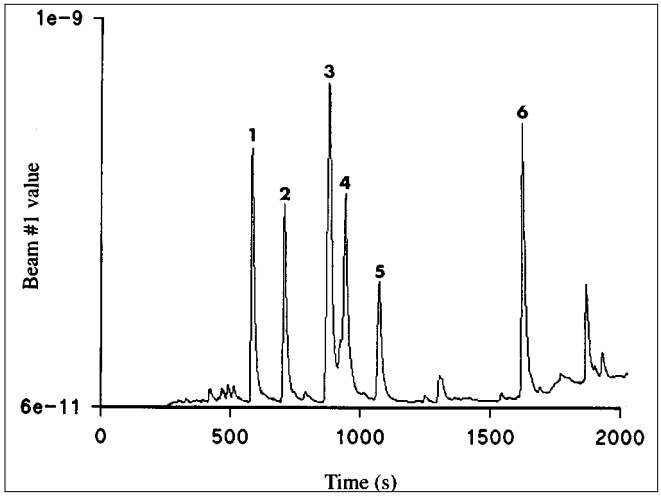

FIG. 1. Gas chromatography–isotope ratio mass spectrometry detection of fatty acid methyl esters from a three-day stool sample. The chromatogram shows the CO_2 peaks obtained from the *m/z* = 44 detector following fatty acid combustion after separation by gas chromatography. Peak 2, 17:0 (internal standard); Peak 6, 23:0 (enrichment standard); Peak 1, 16:0; Peak 3 18:0; Peak 4 18:1; and Peak 5, 18:2.

DISCUSSION

The chromatographic system described here has been utilized to follow the gastrointestinal handling of a labeled lipid substrate. These studies have also shown that GC–IRMS methodology offers good precision and low variability for the determination of ^{13}C abundance in long-chain fatty acids. Values for these parameters were found to be consistent with those specified by the manufacturers, i.e., precision levels of approximately 0.3‰following repeated injections (10) and consistent with those described by others using different GC–IRMS systems (8,11,12). In addition, GC–IRMS has been described as being approximately 1000 times more sensitive than conventional GC–MS in selected ion mode (7). This increase in sensitivity is of great benefit when using stable-isotopes as they are often expensive, may only be available in small quantities, or may be present in low concentrations in test materials.

When establishing the nature of the label, we were careful to ensure that all of the ^{13}C label in feces could be accounted for, this being required for accurate balance studies. By extracting lipid with prior acidification of the fecal sample and showing that no label was associated with the residue following extraction, we demonstrated that the label was only asso-

TABLE 1
Continuous Flow–Isotope Ratio Mass Spectrometry Analysis of the Enrichment Levels of Four Separate Enriched Stool Samples[a]

Extraction method	Sample enrichment (‰^{13}C vs. PDB)	
Acidified solvent extraction	Solvent wash	Remaining residue
Sample 1	+565.5 ± 20.8	−20.0 ± 0.9
Sample 2	+545.4 ± 17.0	−19.8 ± 1.6
Nonacidified solvent extraction		
Sample 1	+244.3 ± 11.6	+107.2 ± 5.1
Sample 2	+209.5 ± 46.0	+166.0 ± 2.7

[a]Treated with and without HCl prior to lipid extraction (mean ± SD, n = 5); PDB, pee dee belemnite; ‰, delta per mil (parts per thousand).

TABLE 2
Mean and SD Values of Enrichments Obtained by Gas Chromatography–Isotope Ratio Mass Spectrometry of Major FAME[a]

	Stool ^{13}C (‰^{13}C vs. PDB)							
	16:0		18:0		18:1		18:2	
Stool	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Baseline	−32.66	0.5	−33.96	2.4	−35.43	0.9	−37.30	1.9
Enriched	+268.6	8.0	−35.76	0.3	−32.27	0.7	−38.56	2.3

[a]Isolated from baseline and enriched stool samples following ingestion of labeled [1-13C]palmitic acid (triplicate injections). FAME, fatty acid methyl esters. See Table 1 for other abbreviation.

ciated with the lipid component of feces. The reason for the label being detected in fecal residue without its prior acidification was likely to be due to the formation of nonextractable fatty acid salts. The acidification step hydrolyzed these compounds, releasing the fatty acid component into the solvent extraction medium (13).

It has been demonstrated for the first time that the nature of ^{13}C enrichment following oral administration of the labeled [1-^{13}C]palmitic acid was primarily restricted to the labeled species consumed by the subject as extracted in the feces. This suggests that there was minimal metabolism of the fatty acid within the gastrointestinal tract and implies that the substrate was either absorbed or excreted intact. It also indicates that our presupposition in a previous paper (6) concerning the gastrointestinal handling of the labeled palmitic acid was appropriate; however, this methodology needs to be substantiated over a larger subject group. Although analysis was not performed on fresh samples, there was no evidence to suggest that a transfer of label to other fatty acids present within the sample could have taken place during storage.

The high enrichment levels obtained suggest that less label could have been administered without compromising analytical precision, since precision degrades at high enrichments (see Table 1). Nevertheless the calibration curve was found to be linear over the range of enrichments measured. Furthermore, the curve could also be used to provide a quantitative measure of the concentration of the labeled species, a vital component for any balance study. The curve was found to be of a similar order to that obtained by Binnert and coworkers (8), who also utilized [1-^{13}C]palmitic acid (99 APE ^{13}C) as a labeled substrate.

Chromatography in GC–IRMS is subject to more overlaps than conventional GC. This is due to the specific requirements such as furnaces and dryers, which convert the chemical into the required gaseous form and also purify it before introduction into the ion source. The large number of connections between the chromatography column and the detector influence peak shape and generally reduce chromatographic efficiency. Thus, $m/z = 44$ chromatograms are much broader than those obtained with work by GC–FID. The peak of interest in this particular study was relatively well separated from any other detected fatty acids, as confirmed by GC–FID analysis. Furthermore, analysis also demonstrated that there was minimal carryover of highly elevated signals as detected in neighboring FAME (see Table 2).

In conclusion, the GC–IRMS methodology described in this paper offers considerable potential for the analysis of the gastrointestinal handling of lipid substrates in human studies. It has been demonstrated that following oral administration of [1-^{13}C]palmitic acid the nature of the label within the stool was primarily associated with the lipid extract and more specifically confined to the form of the administered labeled fatty acid species.

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COMMUNICATION

Dietary Docosahexaenoic Acid as a Source of Eicosapentaenoic Acid in Vegetarians and Omnivores

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ABSTRACT: The utilization of dietary docosahexaenoic acid (DHA; 22:6n-3) as a source of eicosapentaenoic acid (EPA; 20:5n-3) *via* retroconversion was investigated in both vegetarians and omnivores. For this purpose, an EPA-free preparation of DHA was given as a daily supplement (1.62 g DHA) over a period of 6 wk. The dietary supplement provided for a marked increase in DHA levels in both serum phospholipid (from 2.1 to 7.1 mol% in vegetarians and 2.2 to 7.6 mol% in omnivores) and platelet phospholipid (from 1.1 to 3.4 mol% in vegetarians and 1.4 to 3.9 mol% in omnivores). EPA levels rose to a significant but much lesser extent, while 20:4n-6, 22:5n-6, and 22:5n-3 all decreased. Based on the serum phospholipid data, the retroconversion of DHA to EPA *in vivo* was estimated to be 9.4% overall with no significant difference between omnivores and vegetarians.

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Previous animal and *in vitro* studies in isolated rat liver cells have demonstrated that docosahexaenoic (DHA; 22:6n-3) can be retroconverted to eicosapentaenoic acid (EPA; 20:5n-3) (1–3). Human studies (4,5) using supplemented fish oil-derived preparations rich in DHA have supported the *in vivo* retroconversion of DHA to EPA based on the appearance of EPA in plasma phospholipid (4,5) as well as platelet phospholipid (5). However, the DHA concentrates employed in these studies contained some residual EPA (4,5). The 6-d study by Von Schacky and Weber (4) observed a rise in EPA in plasma, but not platelet, phospholipid following DHA ingestion. On the other hand, in the study by Sanders and Hinds (5), the EPA rise in plasma as well as platelet phospholipid occurred with a dietary DHA concentrate having 22% DHA and 8.5% EPA. Further support for the *in vivo* retroconversion of DHA to EPA was published recently by Brossard *et al.* (3) after giving a single dose of ^{13}C-DHA. In addition to the importance of DHA in brain and retinal phospholipid for neuronal functioning and optimal visual performance, respectively (reviewed in Ref. 6), DHA levels in serum/plasma phospholipid (7,8) and EPA levels in platelet phospholipid (9) have been inversely correlated with cardiovascular disease. The primary purpose of the present study was to evaluate the potential for an EPA-free preparation of DHA (DHASCO™; Martek Biosciences Corp., Columbia, MD: derived from an algal source) to provide for EPA as derived *via* retroconversion when the DHA supplement was consumed by vegetarians and omnivores over a 6-wk period, and the fatty acid composition of both plasma and platelet phospholipid was measured. The inclusion of vegetarian subjects in this study was of interest since they consume very minor amounts of EPA/DHA relative to omnivores (10), and various studies have shown that vegetarians have lower serum and/or platelet levels of DHA (11–14). Thus, a comparison of the estimated potential for human retroconversion of DHA to EPA in vegetarians vs. omnivores was of added interest.

MATERIALS AND METHODS

Subjects and experimental design. The subjects were 20 healthy persons [12 vegetarian (6 female, 6 male) and 8 omnivore (4 female, 4 male)] selected from the Guelph community. The vegetarians reported having no meat, poultry, or fish for a period of at least 6 mon. Approval for this study was granted by the Human Ethics Committee of the University of Guelph, and written informed consent was obtained from each subject. Both groups (vegetarians and omnivores) consumed 9 capsules (500 mg each) per day, with meals, of an algae-derived triglyceride oil (DHASCO™) (total 1.62 g DHA/day as measured by quantitative gas–liquid chromatography). The fatty acid composition of the DHA supplement is given in Table 1. Each group consumed the capsules for a period of 42 d beginning on day 0. After 42 d of capsule ingestion, both groups completed a washout period for 21 d during which there was no supplementation. Subjects were weighed on each visit (days 0, 21, 42, 63), and height was measured at entry. Subject characteristics (n = 20) at entry were age (26.8 ± 1.6 y; mean ± SE), weight (67.5 ± 3.4 kg), height (1.73 ± 0.03 m), and body mass index (22.5 ± 1.2 kg/m^2) with no significant differences in these parameters between the groups ($P > 0.05$). The overall weight of the subjects was not significantly affected throughout the supplementation period in either group. All subjects completed the study. Compliance was monitored by a capsule count at the end of the study as well as by determining the fatty acid composition of serum and platelet phospholipid at 3 and 6 wk.

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Abbreviations: ACD, acid citrate dextrose; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

TABLE 1
Fatty Acid Composition of DHASCO™ Capsules[a]

Fatty acids	Total fatty acids (wt%)
8:0	0.05
10:0	1.53
12:0	8.50
13:0	0.01
14:0	21.0
14:1	0.21
16:0	15.8
16:1	1.63
18:0	0.44
18:1	11.2
18:2n-6	0.74
20:5n-3 (EPA)	n.d.
22:5n-3	0.27
22:6n-3 (DHA)	38.6
24:0	0.04
Total saturated	47.3
Monounsaturated	13.1
n-6 Polyunsaturated	0.74
n-3 Polyunsaturated	39.6
n-6/n-3 Ratio	0.02

[a]n.d., not detected; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Blood collection. At day 0 (presupplementation), and days 21 and 42 (supplementation) and day 63 following a 21-d washout, blood was collected by antecubital venipuncture into siliconized tubes (containing no anticoagulant or the anticoagulant ACD [2.5% Na citrate, 2% dextrose, 1.4% citric acid (all from Fisher Chemicals, Nepean, Canada)]. In the tube without anticoagulant, whole blood was centrifuged at 1250 × *g* for 15 min to obtain serum. Serum was used for measurement of the fatty acid contents of total phospholipid. Serum was stored at −20°C until all samples were collected and thawed just before lipid analysis. In the tube containing ACD, washed platelet suspensions were prepared according to the method of Turini *et al.* (15) and stored at −20°C until analysis of total platelet phospholipid.

Fatty acid analysis of phospholipid. The fatty acid compositions of total phospholipid from serum and washed platelet suspensions were determined following lipid extraction, thin-layer chromatography, transmethylation, and gas–liquid chromatography by procedures similar to those previously described (16,17). Gas–liquid chromatography of the fatty acid methyl esters was performed using a Varian 3800 gas chromatograph (Palo Alto, CA) with a 30 m DB-23 capillary column (0.32-mm internal diameter).

Statistical analysis. All data is reported as mean ± SEM. Data was analyzed by Student's unpaired *t*-test. Significance is reported if $P < 0.05$.

RESULTS AND DISCUSSION

Table 2 shows the levels of fatty acids (mol%) in the total phospholipid of human serum before and after supplementation with DHA. Serum total phospholipid fatty acid profiles at entry, including EPA and DHA levels, were not significantly different between the two groups. Changes were seen in various n-3 and n-6 fatty acids after 3 wk of DHA supplementation and those neared maximal changes in some, but not all, cases when compared to the 6-wk data. Relative rises (at 6 wk) in the DHA content (by 247% overall in vegetarians and 240% in omnivores) and the EPA content (by 122% overall in vegetarians and 58% in omnivores) over baseline occurred with DHA supplementation. This was coupled with a rise in the DHA/AA (arachidonic acid; 20:4n-6) ratio (by 414% overall in vegetarians and 318% in omnivores) and the EPA/AA ratio (by 228% in vegetarians and 94% in omnivores). AA (−32 and −19%) and the n-6/n-3 fatty acid ratio (−62 and −60%) decreased in vegetarians and omnivores, respectively. In contrast to the other n-3 fatty acids, the levels

TABLE 2
Fatty Acid Composition (mol%) of Total Phospholipid in Human Serum Before and After Supplementation with DHA[a]

	Omnivore ($n = 8$)				Vegetarian ($n = 12$)			
	Week 0	Week 3	Week 6	Week 9	Week 0	Week 3	Week 6	Week 9
16:0	30.6 ± 0.4	31.5 ± 1.0	32.0 ± 0.5	31.6 ± 0.7	29.3 ± 0.7	30.4 ± 0.5	30.6 ± 0.4	30.9 ± 0.4
18:0	11.9 ± 0.4	12.2 ± 0.5	11.9 ± 0.8	12.4 ± 0.7	12.7 ± 0.4	12.4 ± 0.4	12.2 ± 0.3	12.7 ± 0.3
18:1	12.4 ± 0.4^{a}	11.3 ± 0.4^{b}	11.0 ± 0.4^{b}	11.3 ± 0.3^{b}	13.2 ± 0.6^{a,c}	12.3 ± 0.7^{a,b}	11.7 ± 0.4^{b}	14.1 ± 0.5^{c}
18:2n-6	22.3 ± 0.9^{a}	19.9 ± 0.6^{b}	19.9 ± 0.5^{b}	21.7 ± 0.7^{a,b}	22.1 ± 0.7^{a}	21.5 ± 0.7^{a,b}	21.8 ± 0.7^{a,b}	22.7 ± 0.60^{a}
18:3n-3	0.37 ± 0.03^{a}	0.27 ± 0.03^{a,b}	0.25 ± 0.02^{b}	0.27 ± 0.03^{a,b}	0.25 ± 0.03^{b}	0.23 ± 0.03^{b}	0.23 ± 0.03^{b}	0.29 ± 0.06^{a}
20:3n-6	2.6 ± 0.2^{a}	2.0 ± 0.2^{b}	2.0 ± 0.2^{b}	2.6 ± 0.2^{a}	2.9 ± 0.2^{a}	2.0 ± 0.1^{b}	1.9 ± 0.2^{b}	2.6 ± 0.2^{a}
20:4n-6 (AA)	8.5 ± 0.5^{a}	7.5 ± 0.3^{a,b}	6.9 ± 1.3^{b,c}	7.4 ± 0.5^{a,b}	9.0 ± 0.4^{a}	6.8 ± 0.3^{b,c}	6.1 ± 0.2^{c}	7.4 ± 0.5^{a,b}
20:5n-3 (EPA)	0.73 ± 0.10^{a}	1.00 ± 0.10^{b}	1.16 ± 0.10^{b}	0.74 ± 0.06^{a}	0.54 ± 0.07^{a}	0.96 ± 0.09^{b}	1.19 ± 0.18^{b}	0.69 ± 0.0^{a}
22:4n-6	0.25 ± 0.07^{a}	0.20 ± 0.01^{a}	0.14 ± 0.01^{a,b}	0.21 ± 0.02^{a}	0.34 ± 0.04^{a}	0.11 ± 0.02^{b}	0.14 ± 0.01^{b}	0.14 ± 0.03^{b}
22:5n-6	0.17 ± 0.04^{a}	0.08 ± 0.02^{a,c}	0.06 ± 0.01^{b,c}	0.11 ± 0.03^{a,c}	0.25 ± 0.04^{a}	trace^{b}	trace^{b}	0.06 ± 0.02^{b,c}
22:5n-3	0.68 ± 0.11^{a}	0.42 ± 0.05^{b,c,d}	0.37 ± 0.04^{c}	0.55 ± 0.07^{a,d}	0.79 ± 0.06^{a}	0.34 ± 0.05^{b}	0.37 ± 0.05^{b,c}	0.52 ± 0.07^{a,d}
22:6n-3 (DHA)	2.2 ± 0.2^{a}	6.8 ± 0.3^{b}	7.6 ± 0.3^{b}	4.0 ± 0.3^{c}	2.1 ± 0.2^{a}	6.8 ± 0.3^{b}	7.1 ± 0.4^{b}	4.2 ± 0.3^{c}
n-6/n-3	7.8 ± 0.4^{a}	3.5 ± 0.1^{b}	3.1 ± 0.1^{b}	5.6 ± 0.2^{c}	9.4 ± 0.6^{d}	3.7 ± 0.2^{b}	3.4 ± 0.2^{b}	5.9 ± 0.3^{c}
EPA/AA ratio	0.09 ± 0.01^{a}	0.13 ± 0.01^{b}	0.17 ± 0.01^{c}	0.10 ± 0.01^{a}	0.06 ± 0.01^{a}	0.15 ± 0.01^{b}	0.20 ± 0.02^{c}	0.09 ± 0.01^{a}
DHA/AA ratio	0.26 ± 0.03^{a}	0.91 ± 0.03^{b}	1.10 ± 0.04^{c}	0.54 ± 0.03^{d}	0.23 ± 0.03^{a}	1.00 ± 0.10^{b}	1.16 ± 0.13^{c}	0.57 ± 0.06^{d}

[a]Values are reported as mean ± SEM. Similar superscripts within individual rows are not significantly different (within and between groups). Lipids were analyzed according to the methods referenced in 16,17; AA, arachidonic acid; see Table 1 for other abbreviations. Significance is reported if $P < 0.05$.

of 22:5n-3 were also lowered (by –53 and –46% in vegetarians and omnivores, respectively) as were those of 22:5n-6. By week 9 (following 3 wk of DHASCO™ withdrawal), DHA levels, as well as n-6/n-3 EPA/AA and DHA/AA ratios, in the serum phospholipid of both vegetarians and omnivores were returning to presupplementation levels although they were still significantly different from week 0 (Table 2).

The levels of fatty acids (mol%) in platelet phospholipids before and after supplementation with DHA are shown in Table 3. At entry, omnivores had a significantly ($P < 0.05$) higher level of DHA (1.4 vs. 1.1 mol%) than vegetarians. EPA and 22:5n-3 levels were similar between the two groups. The alterations in the n-3 plus n-6 fatty acids noted at 3 wk were near maximal in most cases when compared to the 6-wk values. After 6 wk of DHA supplementation, a rise was noted in the DHA content of platelets (by 218% overall in vegetarians and 193% in omnivores), as well as in the EPA content of platelets (157% in vegetarians and 151% in omnivores). This was coupled with a rise in the DHA/AA ratio (245% in vegetarians and 199% in omnivores) and the EPA/AA ratio (179% in vegetarians and 156% in omnivores). A decrease in AA (–8% in vegetarians) and the n-6/n-3 ratio (–44% in vegetarians and –41% in omnivores) was noted. Following the pattern in serum phospholipid, there was a decrease in 22:5n-3 levels (–61% in vegetarians and –57% in omnivores) as well as in 22:5n-6. Most fatty acids approached (but not to completion) baseline values after a 3-wk washout period (Table 3).

Table 4 shows the net mol % increase in DHA (Δ DHA) and EPA (Δ EPA) in the serum and platelet phospholipids of vegetarians and omnivores. After 6 wk of consumption of DHA capsules, DHA levels in total phospholipids of serum increased from 2.1 mol% in vegetarians to 7.1% (net 5.1 mol% increase) and from 2.2% in omnivores to 7.6% (net 5.4 mol% increase). In platelets, DHA values increased from 1.1 mol% to 3.4 mol% (net 2.3 mol% increase) in vegetarians and from 1.4 to 3.9% (net 2.6 mol% increase) in omnivores. In vegetarians, EPA levels increased by 0.7 mol% in serum and 0.3% in platelets. EPA levels increased by 0.4 mol% in serum and platelets of omnivores. Thus, the total net mol% increase in EPA + DHA (Δ EPA + Δ DHA), after 6 wk of supplementation with DHA, was 5.7% in the serum of vegetarians and 5.8% in the serum of omnivores and 2.6 and 3.0% in platelets of vegetarians and omnivores, respectively. The (net mol% rise in EPA/net mol% rise in EPA + DHA) × 100 can provide an estimated percentage retroconversion of DHA to EPA. Based on this approach (Table 4), the estimated retroconversion of DHA to EPA is 7.4–11.4% (based on serum phospholipid data) and 12.3–13.8% (based on the platelet phospholipid data) with no significant differences between omnivores and vegetarians. Since DHA retroconversion is considered to actively occur in liver (2), and serum phospholipid is derived primarily from hepatic sources, the value of 9.4% (mean of both groups at week 6 based on serum phospholipid data) may be a better estimate of *in vivo* retroconversion in humans. This estimate does not take into consideration possible differences in oxidation of EPA vs. DHA or the levels of these fatty acids in different lipid classes. Furthermore, it is possible that a plateau in the EPA/DHA levels are not completely reached even by 6 wk although the levels of these fatty acids at 3 and 6 wk were not significantly different from each other (Table 2). Our estimated value for retroconversion (9.4%) is much higher than that estimated recently in human subjects by Brossard *et al.* (3) (1.4%); however, this difference may be due to the duration of DHA supplementation (6 wk in our trial vs. a one time dose of ^{13}C-DHA). Interestingly, the latter authors also reported a 9% retroconversion of DHA to EPA based on their rat trial (3). Measures of EPA and DHA in serum/plasma phospholipid have been considered to provide useful biological indicators for EPA/DHA intake and nutritional status (18–20).

TABLE 3
Fatty Acid Composition (mol%) of Total Phospholipid in Human Platelets Before and After Supplementation with DHA[a]

	Omnivore ($n = 8$)				Vegetarian ($n = 12$)			
	Week 0	Week 3	Week 6	Week 9	Week 0	Week 3	Week 6	Week 9
16:0	19.0 ± 0.2^{a}	18.7 ± 0.3^{a}	19.2 ± 0.2^{a}	18.9 ± 0.2^{a}	19.3 ± 0.3^{a,b}	19.9 ± 0.3^{b}	20.1 ± 0.3^{a}	20.1 ± 0.4^{a,b}
18:0	17.2 ± 0.4^{a}	17.8 ± 0.4^{a}	15.9 ± 0.4^{b}	17.7 ± 0.3^{a}	17.2 ± 0.5^{a}	15.9 ± 0.2^{b}	16.5 ± 0.6^{a}	15.9 ± 0.3^{b}
18:1	17.2 ± 0.3	17.6 ± 0.4	17.4 ± 0.5	17.5 ± 0.3	17.5 ± 0.4	17.6 ± 0.5	18.4 ± 0.5	18.1 ± 0.3
18:2n-6	5.7 ± 0.4^{a}	5.6 ± 0.3^{a}	6.4 ± 0.3^{a}	5.8 ± 0.2^{a}	6.1 ± 0.2^{a}	6.9 ± 0.2^{b}	6.9 ± 0.3b	7.1 ± 0.2^{b}
20:3n-6	1.3 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.3 ± 0.1
20:4n-6 (AA)	24.1 ± 0.7^{a}	22.7 ± 0.4^{b}	23.6 ± 0.5^{a}	23.4 ± 0.4^{a}	22.4 ± 0.7^{a,b}	22.3 ± 0.4^{b}	20.6 ± 0.7c	23.2 ± 0.4^{a,b}
20:5n-3 (EPA)	0.28 ± 0.05^{a}	0.52 ± 0.05^{b}	0.69 ± 0.05^{c}	0.44 ± 0.04^{b}	0.20 ± 0.04^{a}	0.45 ± 0.06^{b}	0.52 ± 0.06^{c}	0.35 ± 0.08^{a,b}
22:4n-6	1.9 ± 0.1^{a}	1.3 ± 0.1^{b}	1.1 ± 0.1^{c}	1.5 ± 0.1^{d}	2.1 ± 0.2^{a}	1.1 ± 0.1^{b,e}	1.0 ± 0.1^{c,e}	1.4 ± 0.1^{d}
22:5n-6	0.23 ± 0.04^{a,b}	0.09 ± 0.02^{c}	trace^{d}	0.15 ± 0.03^{c}	0.14 ± 0.04^{a}	trace^{d}	trace^{d}	0.03 ± 0.02^{d}
22:5n-3	1.55 ± 0.21^{a}	0.73 ± 0.06^{b}	0.66 ± 0.04^{b}	0.99 ± 0.06^{c}	1.48 ± 0.10^{a}	0.57 ± 0.05^{d}	0.58 ± 0.07^{b,d}	0.96 ± 0.06^{c}
22:6n-3 (DHA)	1.4 ± 0.1^{a}	3.4 ± 0.2^{b}	3.9 ± 0.2^{c}	2.5 ± 0.1^{d}	1.1 ± 0.1^{e}	3.2 ± 0.1^{b}	3.4 ± 0.2^{b}	2.3 ± 0.1^{d}
n-6/n-3	8.7 ± 0.4^{a}	6.7 ± 0.2^{b}	6.1 ± 0.2^{b}	8.15 ± 0.3^{c}	11.4 ± 0.6^{d}	7.3 ± 0.3^{b}	6.7 ± 0.3^{b}	9.4 ± 0.4^{c}
EPA/AA ratio	0.01 ± 0.00^{a}	0.02 ± 0.00^{b}	0.03 ± 0.00^{c}	0.02 ± 0.00^{b}	0.01 ± 0.00^{a}	0.02 ± 0.00^{b}	0.03 ± 0.00^{c}	0.02 ± 0.00^{b}
DHA/AA ratio	0.06 ± 0.00^{a}	0.15 ± 0.01^{b}	0.17 ± 0.01^{b,c}	0.11 ± 0.01^{d}	0.05 ± 0.00^{a}	0.15 ± 0.02^{b}	0.17 ± 0.02^{c}	0.10 ± 0.01^{d}

[a]Values are reported as mean ± SEM. Similar superscripts within individual rows are not significantly different (within and between groups). Lipids were analyzed according to the methods referenced in 16,17. See Tables 1 and 2 for abbreviations. Significance is reported if $P < 0.05$.

TABLE 4
Effect of DHA Supplementation on the Net Increase in EPA and DHA Levels in Serum and Platelet Phospholipid[a]

	Omnivore (n = 8)		Vegetarian (n = 12)	
Parameter	Serum	Platelet	Serum	Platelet
Δ EPA (mol%)	0.43 ± 0.14[a,b]	0.41 ± 0.07[a,b]	0.65 ± 0.12[b]	0.32 ± 0.05[a]
Δ DHA (mol%)	5.4 ± 0.4[a]	2.6 ± 0.2[b]	5.1 ± 0.3[a]	2.3 ± 0.2[b]
Δ EPA + Δ DHA (mol%)	5.8 ± 0.4[a]	3.0 ± 0.1[b]	5.7 ± 0.3[a]	2.6 ± 0.2[b]
Δ EPA/Δ EPA + Δ DHA (as relative %)	7.4 ± 1.3[a]	13.8 ± 1.6[b]	11.4 ± 1.7[a,b]	12.3 ± 1.8[b]

[a]Values are reported as means ± SEM as a result of 6 wk of DHASCO™ supplementation. Similar superscripts within individual rows are not significantly different. See Table 1 for abbreviations. Significance is reported if $P < 0.05$.

Our 6-wk supplementation trial in vegetarians and omnivores employed an EPA-free preparation of DHA wherein all other n-3 polyunsaturated fatty acids were present at levels less than 0.3%. Our vegetarian subjects exhibited significantly lower levels of DHA in platelet phospholipid relative to omnivores as reported by others (13,14). Unlike previous studies (11–14), the lower EPA/DHA levels in the serum phospholipid of our vegetarian subjects (relative to the omnivores) did not reach statistical significance, perhaps because our subject group of healthy Canadian vegetarians was unlike the subject groups in the other studies (vegans, rheumatoid arthritis patients, people of Asian Indian background). Previous studies in omnivores employing DHA preparations containing residual EPA have shown significant rises of EPA in serum phospholipid (4,5) with (5) or without (4) a corresponding rise in EPA in platelet phospholipid. The study of Sanders and Hinds (5), which showed a rise in EPA in platelet phospholipid over 6 wk, employed a supplemented preparation which contained 22% DHA and 8.5% of residual EPA which does not allow for a calculation of retroconversion in humans. The study by Von Schacky and Weber (4) showed a rise in EPA in serum but not platelet phospholipid when a DHA concentrate was given orally for 6 d to human volunteers. The failure of the latter study to exhibit a rise in EPA in platelet phospholipid, in contrast to our present findings, likely lies with the duration of the study employed (6 d as compared to 42 d in the present investigation). Recent studies have indicated that the hepatic retroconversion of DHA to EPA is a peroxisomal function (2).

It remains to be established whether the rise in DHA and also EPA *via* retroconversion in vegetarian subjects upon consuming an algal source of DHA (DHASCO™) provides any health benefits. Future studies on varying dose levels of DHA in both vegetarians and omnivores likely will be of interest considering the accumulating evidence that DHA is an essential nutrient, in the brain and retina, for neuronal and visual functioning, respectively (6). Furthermore, the dietary intake of EPA/DHA from seafood has been associated with a reduced risk of primary cardiac arrest (21); also, DHA levels in serum phospholipid (7,8) and EPA levels in platelet phospholipid (9) have been inversely correlated with cardiovascular disease.

Interestingly, the dietary DHA supplement suppressed the levels of both 22:5n-6 and 22:5n-3 in both serum and platelet phospholipid. Whether these biochemical changes represent competition at the level of fatty acid esterification into the corresponding cellular phospholipid (at the level of *de novo* phospholipid synthesis or acyl transferase reactions) or other modification of polyunsaturated fatty acid metabolism (effects on 22:5n-6 and 22:5n-3) remains to be studied further.

In conclusion, the present results indicate that an EPA-free concentrate of DHA consumed over a period of 6 wk can significantly enrich the level of DHA as well as EPA in both serum and platelet phospholipid of omnivores and vegetarians. The estimated retroconversion of dietary DHA to EPA based on these studies is 9.4% with no significant differences apparent between omnivores and vegetarians. Future studies using deuterated precursors which study the metabolism and turnover of DHA and its fatty acid products *in vivo* will be of interest in further determinations of the estimated retroconversion as derived from our present study.

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ARTICLE

Phospholipid Biosynthetic Enzymes in Human Brain

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ABSTRACT: Growing evidence suggests an involvement of brain membrane phospholipid metabolism in a variety of neurodegenerative and psychiatric conditions. This has prompted the use of drugs (e.g., CDPcholine) aimed at elevating the rate of neural membrane synthesis. However, no information is available regarding the human brain enzymes of phospholipid synthesis which these drugs affect. Thus, the objective of our study was to characterize the enzymes involved, in particular, whether differences existed in the relative affinity of substrates for the enzymes of phosphatidylethanolamine (PE) compared to those of phosphatidylcholine (PC) synthesis. The concentration of choline in rapidly frozen human brain biopsies ranged from 32–186 nmol/g tissue, a concentration similar to that determined previously for ethanolamine. Since human brain ethanolamine kinase possessed a much lower affinity for ethanolamine (K_m = 460 μM) than choline kinase did for choline (K_m = 17 μM), the activity of ethanolamine kinase *in vivo* may be more dependent on substrate availability than that of choline kinase. In addition, whereas ethanolamine kinase was inhibited by choline, and to a lesser extent by phosphocholine, choline kinase activity was unaffected by the presence of ethanolamine, or phosphoethanolamine, and only weakly inhibited by phosphocholine. Phosphoethanolamine cytidylyltransferase (PECT) and phosphocholine cytidylyltransferase (PCCT) also displayed dissimilar characteristics, with PECT and PCCT being located predominantly in the cytosolic and particulate fractions, respectively. Both PECT and PCCT exhibited a low affinity for CTP (K_m approximately 1.2 mM), suggesting that the activities of these enzymes, and by implication, the rate of phospholipid synthesis, are highly dependent upon the cellular concentration of CTP. In conclusion, our data indicate different regulatory properties of PE and PC synthesis in human brain, and suggest that the rate of PE synthesis may be more dependent upon substrate (ethanolamine) availability than that of PC synthesis.

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The first committed step of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) synthesis is base phosphorylation by choline or ethanolamine kinase. This is followed by the incorporation of CTP to form CDPcholine or CDP-ethanolamine by the enzymes phosphocholine cytidylyltransferase (PCCT) and phosphoethanolamine cytidylyltransferase (PECT), respectively. Although the cytidylyltransferase-catalyzed reaction has been considered the rate-limiting step of the pathway, the activities of the kinases, as well as choline and ethanolamine availability, may also play a regulatory role [for a full discussion see Kent (1)]. CDPcholine and CDP-ethanolamine then react with diacylglycerol (or 1-alkyl-2-acylglycerol) to produce a molecule of phospholipid, reactions catalyzed by choline phosphotransferase and ethanolamine phosphotransferase, respectively. This latter reversible reaction operates close to equilibrium and does not appear to play a regulatory role in the pathway (1).

Abnormal membrane breakdown and/or turnover has been implicated in several human brain disorders including Alzheimer's disease (2,3), cerebral ischemia (4,5), schizophrenia (6–8), and cerebellar ataxia (9,10). This has prompted some investigators to consider the use of inhibitors of membrane breakdown or stimulators of membrane synthesis as potential therapeutic agents in these diseases. One compound in particular, cytidine (5′)-diphosphocholine (CDPcholine or Citicoline), which elevates the rate of brain phospholipid synthesis (11), appears to be effective in the treatment of cerebral ischemia (12,13) and head injury (14), and in ameliorating age-associated memory deficits (15). CDPcholine is completely hydrolyzed to cytidine and choline in the periphery (16,17), and appears to act by increasing levels of these compounds in the brain. However, the relative importance of each hydrolysis product (cytidine or choline) in mediating the beneficial effects of the drug is unknown. To address this question, one must first determine the substrate kinetics of the enzymes involved, which, although available for nonhuman species [reviewed in Kent (1)], are unavailable for the human enzymes. Thus, we have determined some important kinetic properties of the human brain enzymes, in order to better understand how their characteris-

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Abbreviations: PC, phosphatidylcholine; PCCT, phosphocholine cytidylyltransferase; PE, phosphatidylethanolamine; PECT, phosphoethanolamine cytidylyltransferase; TLC, thin-layer chromatography.

tics might relate to the mechanism of action of drugs such as CDPcholine.

METHODS

Materials. Temporal (Brodmann area 21) cortex was dissected from the brains of four neurologically normal subjects age 52 ± 4 yr (mean ± SEM), 10 ± 1 h after death (Table 1). The brains were frozen at autopsy and stored at −80°C. All tissue used was free of gross infarct and tumors, and showed no indication of neurological disease as assessed by neuropathological examination. Biopsied temporal cortex, frozen in liquid nitrogen immediately after removal, was obtained from individuals (age 31 ± 4) who had undergone temporal lobectomy for epileptic seizures (Table 1). Biopsied tissue was from nonspiking regions as determined by electrode electrography (18). Radiochemicals were purchased from New England Nuclear (Boston, MA), Amersham International (Amersham, United Kingdom), and American Radiolabeled Chemicals (St. Louis, MO). All other reagents were obtained from either BDH (Poole, United Kingdom) or the Sigma Chemical Company (St. Louis, MO). Thin-layer chromatography (TLC) plates were 20-cm Whatman silica gel 150A plates (Maidstone, England) (type LK5D) with linear-K loading strip.

Tissue preparation. Tissue was disrupted by sonication in 5 vol of 1 mM EDTA, 1 mM EGTA, 50 mM HEPES, pH 7.4, and cytosolic and particulate fractions prepared as described previously (18).

PECT activity assay. Approximately 50 µg cytosolic or 80 µg of particulate protein was incubated for 1 h at 37°C in a buffer (final volume 50 µL) containing 15 mM magnesium chloride, 10 mM CTP, 1 mM ^{3}H-phosphoethanolamine (2 nCi/nmol), and 100 mM HEPES, pH 7.5, with alterations as described in the text. Following incubation, water-soluble products were extracted by the sequential addition of 200 µL chloroform/methanol [1:2 (vol/vol)], 50 µL chloroform, and 50 µL water (19). A 150 µL aliquot of the aqueous phase was dried by vacuum centrifugation, the residue resuspended in 30 µL of water containing 5 µg of ethanolamine, 10 µg of phosphoethanolamine, and 10 µg of CDPethanolamine, and applied to a silica gel TLC plate. Following development in 96% ethanol/water/25% ammonia solution [75:25:3 (by vol)] (20), amines were visualized with 0.2% ninhydrin and the spot corresponding to CDPethanolamine scraped into scintillation fluid and radioactivity quantified.

PCCT activity assay. This was carried out as for PECT except that (i) 225 µg cytosolic or 50 µg particulate protein was used, (ii) phosphoethanolamine was replaced by 1 mM ^{14}C-phosphocholine (4 nCi/nmol) for the cytosolic enzyme, or 2 mM ^{14}C-phosphocholine (1 nCi/nmol) for the particulate enzyme, with alterations as described in the text, (iii) following drying, the residue was resuspended in 30 µL of water containing 100 µg of choline, 500 µg of phosphocholine, and 800 µg of CDPcholine, and (iv) the TLC plate developed in 96% ethanol/water/25% ammonia solution [60:40:3 (by vol)], followed by visualization with iodine vapor, and quantification of the radioactivity in the CDPcholine spot.

Choline kinase. Approximately 30 µg of cytosolic protein was incubated for 1 h at 37°C in a buffer (final volume 100 µL) containing 10 mM magnesium chloride, 10 mM ATP, 100 mM HEPES, pH 8.0, 200,000 dpm ^{14}C-choline, and varying choline concentration, and other modifications, as described in the text. Following incubation, water-soluble products were extracted, and phosphocholine quantified by ion exchange chromatography as described previously (19). Column recovery was corrected for by running radiolabeled phosphocholine standards in each assay.

Ethanolamine kinase. This was carried out as for choline kinase except that 100 µg of cytosolic protein was used, and ethanolamine replaced choline. Isolation of phosphoethanolamine was accomplished by modifying the procedure used for isolating phosphocholine (19), namely, the column was washed first with 2 mL of water, and phosphoethanolamine was eluted with 8 mL of water, as determined by comparision with a radiolabeled phosphoethanolamine standard. This procedure resulted in a recovery of approximately 60% for phosphoethanolamine, which was corrected for by running radiolabeled phosphoethanolamine standards in each assay. Recovery of phosphoethanolamine was not affected by the co-application of 1 µmol phosphocholine or choline to the column, amounts in excess of that employed in the inhibitor studies of ethanolamine kinase.

Determination of tissue choline content. The choline content of brain tissue was determined as described previously (2).

Nonlinear curve fitting. This was accomplished using the "Prism" computer software program (Graphpad, San Diego, CA), which uses an iterative procedure to fit dual and single K_m models to data. Both K_m and V_{max} values were left unconstrained and were determined automatically by the program. The program was also used to statistically compare the goodness-of-fit of each model.

pH sensitivity. The buffers used at each pH were 5.0–6.5, MES; 7.0–8.0, HEPES; 8.5–10, borate.

Effect of adding lipid to cytosolic protein. Homogenates were separated into cytosolic and particulate fractions as described above. The cytosolic fraction was then placed on ice

TABLE 1
Human Brain Tissue Used in This Study[a]

Subject	Sex	Age (yr)	Post-mortem interval (h)	Cause of death
Biopsy 1	F	19	NA	NA
Biopsy 2	M	32	NA	NA
Biopsy 3	M	28	NA	NA
Biopsy 4	M	34	NA	NA
Biopsy 5	F	41	NA	NA
Autopsy 1	M	41	8	Multiple injuries
Autopsy 2	M	65	11	Myocardial infarction
Autopsy 3	F	54	14	Drowning
Autopsy 4	M	48	9	Multiple injuries

[a]Tissue was obtained from the temporal cortex; NA, not applicable.

TABLE 2
Enzyme Activities in Biopsied and Autopsied Human Brain[a]

Enzyme	Fraction	Biopsied (nmol/h/mg)	Autopsied (nmol/h/mg)
EK	Cytosol	22 ± 1	20 ± 1
CK	Cytosol	27 ± 2	29 ± 3
PECT	Cytosol	38 ± 3	35 ± 2
	Particulate	5 ± 1	4 ± 2
PCCT	Cytosol	0.7 ± 0.5	0.8 ± 0.2
	Particulate	42 ± 3	45 ± 4

[a]Values are mean ± SEM activities assayed in preparations of four autopsied or four biopsied (Biopsies 1–4; see Table 1) human temporal cortices. Ethanolamine kinase (EK) and choline kinase (CK) activities were determined using 2 mM ethanolamine and 2 mM choline, respectively. Phosphoethanolamine cytidylyltransferase (PECT) and phosphocholine cytidylyltransferase (PCCT) were assayed as described in the Methods section. No significant differences were detected between activity in biopsied and autopsied brain (two-tailed Student's *t*-test; $P > 0.4$).

while lipid was extracted form the particulate fraction (21). The lipid extract was then resuspended by sonication in a small volume of water, and added to an aliquot of the cytosolic fraction in a proportional manner. The cytosolic fractions with and without added lipid were sonicated on ice for 1 min, and left on ice for a further 30 min before use.

Proteins were assayed using the Bio-rad protein assay (Richmond, CA) according to the manufacturer's instructions.

RESULTS

Enzyme activities in autopsied and biopsied human brain. The reaction rate of each enzyme in human brain homogenates was linear with respect to time for at least 1 h at 37°C, and up to at least 200 µg of cytosolic protein for ethanolamine and choline kinase, and 300 µg of cytosolic or particulate protein for PECT and PCCT (data not shown). Activity of all enzymes was not significantly different in homogenates of autopsied and biopsied homogenates of human brain (Table 2), indicating that each activity was stable post mortem and suitable for futher study in autopsied tissue.

Choline content of biopsied human brain. The levels of choline in biopsied samples of three human temporal cortices were 40 nmol/g in Biopsy 1 (see Table 1), 186 nmol/g in Biopsy 2, and 32 nmol/g in Biopsy 5. Assuming a water content of 90% tissue weight, these values correspond to aqueous concentrations of 44 µM, 206 µM, and 36 µM, respectively. Choline concentration was not assayed in the remaining biopsies due to tissue limitations.

Ethanolamine and choline kinase. Both choline and ethanolamine kinase activities were localized almost exclusively (>90%) in the cytosolic fraction, and possessed a pH optimum of approximately 8.0 (data not shown). Both enzymes exhibited Michaelis–Menten kinetics for ATP up to a concentration of 10 mM, with higher substrate concentrations being inhibitory (Fig. 1B). As shown in Table 3, the K_m of ATP for ethanolamine ki-

TABLE 3
Kinetic Parameters for Enzymes of the Phospholipid Biosynthetic Pathway of Human Brain[a]

Enzyme	Fraction	Substrate	Co-substrate	K_m (µM)	V_{max} (nmol/h/mg)
EK	Cytosol	Eth (50–5000 µM)	10 mM ATP	460 ± 20 (500 ± 50)	33 ± 2
		ATP (200–10000 µM)	2 mM Eth	2200 ± 320	30 ± 4
CK	Cytosol	Ch (5–5000 µM)	10 mM ATP	1. 6 ± 0.1[b] (10 ± 0.3)[b]	16 ± 6
				2. 1270 ± 210 (1360 ± 220)	30 ± 8
		ATP (200–10000 µM)	10 µM choline	580 ± 50	6 ± 1
			2 mM choline	620 ± 70	34 ± 3
PECT	Cytosol	PEth (20–5000 µM)	10 mM CTP	204 ± 4 (2200 ± 6)	47 ± 5
		CTP (200–10000 µM)[c]	1 mM PEth	1340 ± 330	44 ± 8
	Particulate	PEth (20–5000 µM)	10 mM CTP	145 ± 30	7 ± 1
PCCT	Cytosol	PCh (20–5000 µM)	10 mM CTP	141 ± 8 (158 ± 8)	1.0 ± 0.07
		CTP (200–10000 µM)[c]	1 mM PCh	1210 ± 200	0.9 ± 0.1
	Particulate	PCh (20–5000 µM)	10 mM CTP	310 ± 30	58 ± 11

[a]Values are mean ± SEM of each parameter measured the indicated cell fraction of 3–4 autopsied human temporal cortices analyzed in independent experiments. Abbreviations: EK, ethanolamine kinase; CK, choline kinase; Eth, ethanolamine; Ch, choline; PCh, phosphocholine; PEth, phosphoethanolamine. K_m and V_{max} values were calculated using 7–11 substrate concentrations per subject, and derived by Eadie–Hofstee transformation, except for choline with CK for which nonlinear curve fitting was used to derive dual K_m values (see the Results section). The values in parentheses following the K_m values are the same parameter recalculated using substrate concentrations corrected approximately for the presence of endogenous substrate, the values used being: choline, 250 µM; phosphocholine, 350 µM; ethanolamine, 250 µM; phosphoethanolamine, 250 µM (Ref. 2).

[b]When the K_m for choline and the V_{max} of CK are calculated by Eadie–Hofstee transformation using choline concentrations up to 200 µM (i.e., the first plateau in the substrate/activity relationship, see Fig. 1), the K_m is 17 ± 3 µM (24 ± 3 µM corrected for endogenous choline), and V_{max} is 33 ± 5 nmol/h/mg.

[c]Kinetics were not derived for the particulate fraction owing to the high CTPase activity of this fraction (Ref. 40).

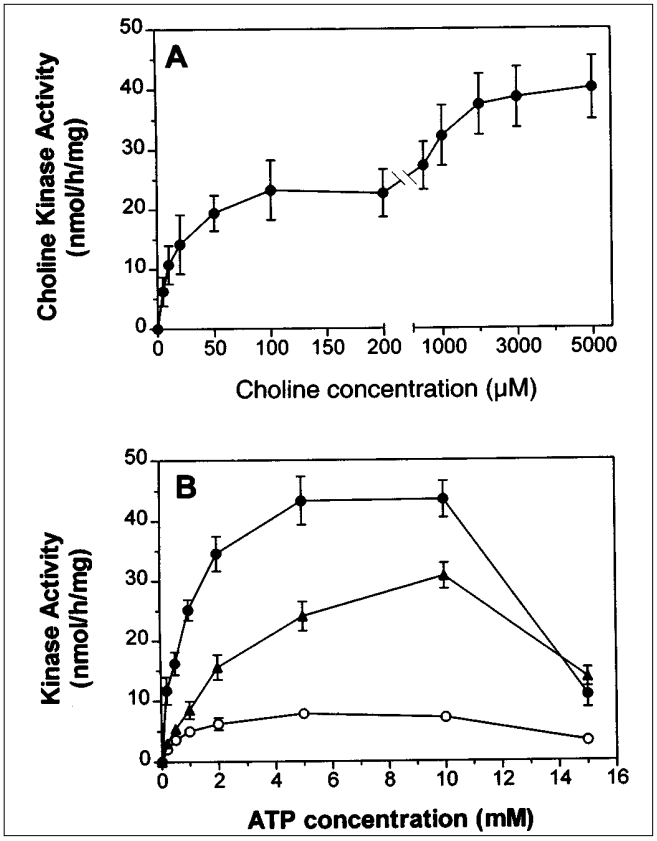

FIG. 1. Selected kinetic curves. (A) Cytosolic choline kinase under varying choline concentrations (note break in x axis) using an ATP concentration of 10 mM, and (B) ethanolamine kinase (▲) using 2 mM ethanolamine, and choline kinase using 1.2 mM (●) or 10 μM (○) choline, under varying ATP concentrations. Each point gives the mean activity of 3–4 autopsied human temporal cortices analyzed in separate experiments. All substrate concentrations shown were used in each experiment, with each brain preparation analyzed giving qualitatively similar results. Error bars indicate the SEM.

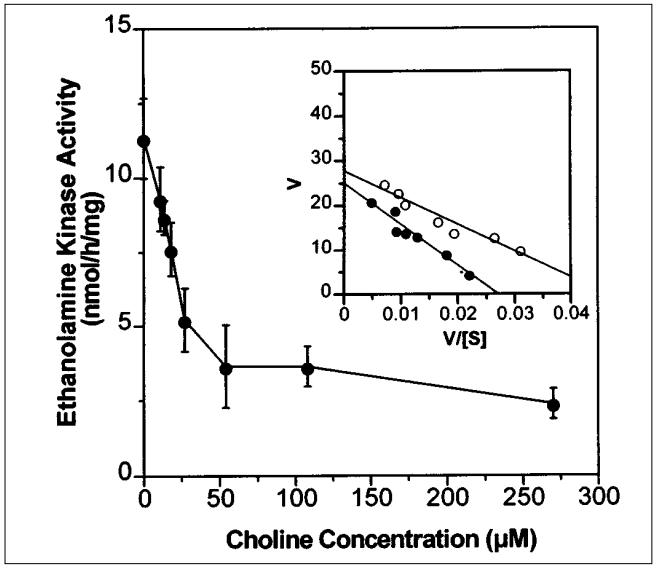

FIG. 2. Effect of choline on ethanolamine kinase activity. Main figure: Ethanolamine kinase was assayed using 0.5 mM ethanolamine (approximate K_m concentration) and 10 mM ATP, under varying choline concentrations. Each point gives the mean activity of three autopsied human temporal cortices analyzed in separate experiments. Bars indicate the SEM. Inset: Eadie–Hofstee plot of a representative experiment in which ethanolamine kinase was assayed under varying ethanolamine concentrations (50–5000 μM) and 10 mM ATP in the presence (●) or absence (○) of 15 μM choline.

nase (2200 ± 320 μM) was somewhat higher than that for choline kinase (620 ± 70 μM). Ethanolamine kinase also displayed Michaelis–Menten kinetics with respect to ethanolamine, which possessed a K_m of approximately 0.5 mM for the enzyme (Table 3). In contrast, choline kinase possessed two distinct saturation points, the first at approximately 100–200 μM choline, and the second at approximately 2–5 mM choline (Fig. 1A). Analysis of these experiments using nonlinear curve fitting produced a better fit using a dual K_m model, with K_m values of approximately 10 μM and 1.2 mM (Table 2), than that produced using a single K_m model (F-test; $P < 0.005$; $n = 4$). The use of high (1.2 mM) or low (10 μM) choline concentrations did not affect the pH optimum of choline kinase (data not shown), or the K_m of ATP for the enzyme (Table 3).

Choline was a potent inhibitor of ethanolamine kinase, with 50 μM choline inhibiting the enzyme by 65 ± 7% ($n = 3$) using 500 μM ethanolamine as substrate (i.e., substrate concentration 10 times that of the inhibitor) (Fig. 2). Choline appeared to act as a competitive inhibitor since 10 μM choline raised the K_m of ethanolamine kinase to 940 ± 160 μM from 460 ± 20 μM (no choline added) ($n = 3$), but did not alter V_{max} (Fig. 2). However, it should be noted that at higher choline concentrations (>50 μM), inhibition of ethanolamine kinase activity was somewhat less than that theoretically predicted by a competitive inhibition model (Fig. 2). Employing the formula $K_m' = K_m (1 + [I]/K_i)$ (22), an approximate K_i of 10 ± 3 μM can be calculated for choline. In contrast, choline kinase was not inhibited by ethanolamine added at concentrations between 0.5 and 10 times that of choline (data not shown). The lack of inhibition of choline kinase by ethanolamine was observed at choline concentrations of 10 μM (up to 100 μM ethanolamine added) and 1.2 mM (up to 12 mM ethanolamine added).

Ethanolamine kinase was also inhibited by phosphocholine, though less potently than that observed using choline, with 200 μM phosphocholine causing 34 ± 6% inhibition using 2 mM ethanolamine as substrate (i.e., substrate concentration 10 times that of the inhibitor) (Fig. 3A). Higher concentrations of phosphocholine also inhibited choline kinase (Fig. 3A). Again, it appeared that inhibition was competitive since although phosphocholine did not alter the V_{max} of either enzyme, 100 μM phosphocholine increased the K_m of ethanolamine kinase to 1130 ± 190 μM from 460 ± 20 μM in the absence of phosphocholine ($n = 3$; Fig. 3B), while 1 mM phosphocholine raised the K_m of choline kinase to 32 ± 4 μM from 17 ± 3 μM in the absence of phosphocholine ($n = 3$; calculated by Eadie–Hofstee transformation using choline

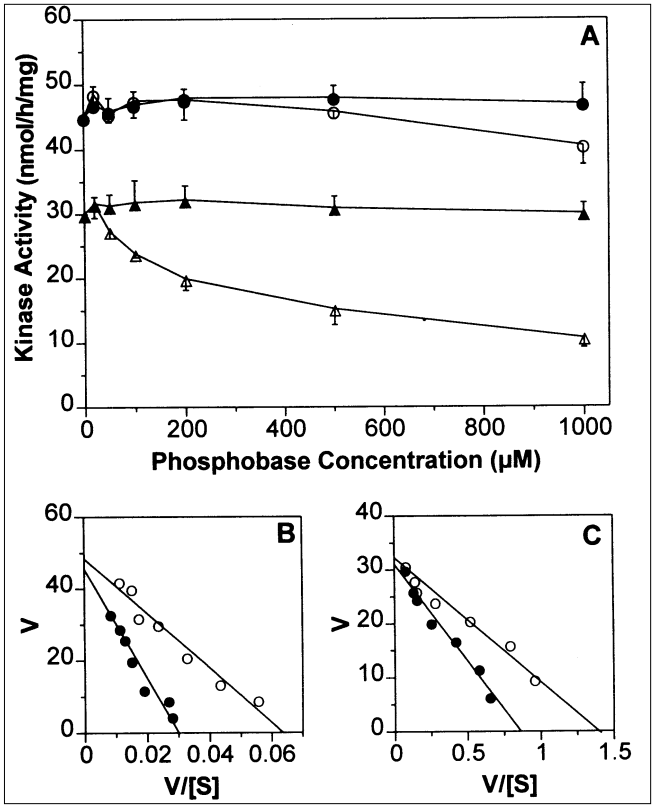

FIG. 3. Effect of phosphobases on choline and ethanolamine kinase activity. (A) Cytosolic choline kinase (●,○) and cytosolic ethanolamine kinase (▲,△) activities were assayed in the presence of varying concentrations of phosphocholine (△,○) and phosphoethanolamine (▲,●). Each enzyme was assayed using 10 mM ATP and 2 mM choline/ethanolamine. Each point gives the mean activity of three autopsied human temporal cortices analyzed in separate experiments. Bars indicate the SEM. (B) and (C). Eadie–Hofstee plots of a representative experiment in which (B) ethanolamine kinase was assayed under varying ethanolamine concentrations (50–5000 μM) and 10 mM ATP in the presence (●) or absence (○) of 100 μM phosphocholine, and (C) choline kinase under varying choline concentrations (5–200 μM) and 10 mM ATP in the presence (●) or absence (○) of 1 mM phosphocholine.

concentrations up to 200 μM) (Fig. 3C). Approximate K_i values for phosphocholine are 69 ± 12 μM for the inhibition of ethanolamine kinase, and 1133 ± 60 μM for the inhibition of choline kinase. In contrast, phosphoethanolamine, used at concentrations up to 1 mM, did not inhibit the activity of either enzyme under the conditions tested (Fig. 3A).

PECT and PCCT. The majority of PECT activity was found in the cytosolic compared to particulate fractions, with the reverse being true for PCCT activity (see Table 2). Both enzymes possessed a pH optimum of 7.5 (data not shown), and exhibited Michaelis–Menten kinetics for both CTP and the phosphobase (Table 3). The K_m of PE for PECT was similar in both particulate and cytosolic fractions; however, the K_m of PC for PCCT was significantly higher (approximately 2.5-fold) in the particulate compared to cytosolic fractions (Table 3). This did not appear to be due to the absence of lipid in the cytosolic fraction since, when lipid extracted from the particulate fraction was added in a proportional manner to the cytosolic fraction (see the Methods section for details), no significant change in the K_m of phosphocholine for cytosolic PCCT was apparent, although V_{max} increased slightly (K_m of phosphocholine and V_{max} were 157 ± 20 μM and 1.7 ± 0.05 nmol/h/mg, respectively, for cytosol plus lipid, compared to 141 ± 11 μM and 1.0 ± 0.1 nmol/h/mg, respectively, for cytosol minus lipid; $n = 3$).

To investigate the substrate specificity of PECT and PCCT, either phosphocholine or phosphoethanolamine was added to the assay of PECT and PCCT, respectively. The activity of both enzymes was unaltered by the addition of these compounds used at up to 10 times the concentration of substrate (data not shown; CTP was 1.2 mM in all cases, phosphocholine was 150 μM for PCCT, and phosphoethanolamine 150 μM for assaying cytosolic PECT activity and 300 μM for particulate PECT activity). Finally, the addition of 1 mM serine, choline, or ethanolamine did not alter the activity of either PECT or PCCT (data not shown).

DISCUSSION

We have characterized four enzymes central to the synthesis of phospholipids in brain, and have shown some differences in the features of those enzymes involved in PE synthesis compared to those in the PC pathway.

Choline and ethanolamine kinase. The non-Michaelis–Menten relationship between choline concentration and choline kinase activity suggests the presence of either two forms of choline kinase in human brain or two sites on the same enzyme in which catalysis can take place, one with low affinity for choline (millimolar K_m), and another with a high affinity (approximately 10 μM K_m). The relative importance of these two sites depends on the actual concentration of choline present in human brain. Although this value was previously available for autopsied tissue (approximately 250 μM) (2), we have determined, to our knowledge for the first time, the concentration of choline in biopsied human brain. Owing to the short time between tissue dissection and freezing, the abundance of free choline in these tissue samples likely reflects the choline concentration *in vivo* at the time of collection. One biopsy had choline concentrations approximately five times those of the other two, possibly indicating the occurrence of ischemia in the brain tissue from which the biopsy was taken, since ischemia leads to a rapid rise in tissue free choline concentration (42). However, in two biopsies, tissue choline concentration was approximately 40 μM, a value comparable to that found in rodent brain (30–60 μM (23,24). Thus, under normal conditions, human brain choline kinase may be viewed as a single enzyme half maximally active at choline concentrations of approximately 20 μM (see Fig. 1 and Table 3). In this case, assuming an *in vivo* choline concentration of approximately 40 μM, human brain choline kinase will be operating at approximately two times the K_m for choline. Thus, increasing choline levels further, e.g., to 200 μM (see Fig. 1), for example, by the ad-

ministration of choline-containing compounds, e.g., PC, CDPcholine, should result in only a modest rise in flux through this enzyme. While it is not known whether phosphocholine levels rise in human brain in response to choline administration, an elevation of brain phosphocholine levels does occur in an animal model following choline supplementation (43). Assuming similar characteristics of human and rat choline kinases, such observations are consistent with the *in vivo* activity of PCCT being much lower than that of choline kinase (1). In such a scenario, small increases in choline kinase activity would be expected to have a relatively greater effect upon phosphocholine levels. Alternatively, the presence of some form of "channeling" of choline between the choline transporter and choline kinase may take place, occurring in the absence of equilibration with total intracellular pools. Indeed, some evidence exists for the presence of a functional link between individual steps of PC biosynthesis in cultured glioma cells (44).

Perry *et al.* (25) reported that the concentration of ethanolamine in biopsied human brain is in the order of 70 μM, well below its K_m of approximately 500 μM for ethanolamine kinase. Thus, the activity of ethanolamine kinase in human brain may be much more dependent upon cellular levels of its substrate ethanolamine than choline kinase activity is upon cellular choline levels. Indeed, the major limiting factor for PE biosynthesis in rat liver appears to be the availability of ethanolamine (26). The possibility therefore exists that compounds designed to elevate ethanolamine levels in the brain may be more effective at increasing phospholipid synthesis than those which increase choline.

Ethanolamine kinase was found to be inhibited by both choline and phosphocholine at concentrations normally present in the brain [this study and Nitsch (2)]. Thus, in the absence of some form of compartmentation, choline-containing compounds may partly inhibit ethanolamine kinase *in vivo*. The role of such a mechanism is unclear, but may serve to quickly increase the synthesis of PE in times of low choline availability, thus facilitating increased synthesis of PC *via* the methylation pathway. Indeed, in rat liver, choline deficiency results in elevated PE levels in the absence of increased activity *in vitro* of the synthetic enzymes (27,28). Similarly, choline deficiency stimulates the rate of PE synthesis in cultured adrenal medullary cells (29). Our observations also suggest that excessive elevation of choline levels may actually decrease the rate of PE synthesis in brain due to the inhibition of ethanolamine kinase, as appears to occur in the heart (30).

Cytidylyl transferase. The PECT and PCCT activities of human brain are most clearly differentiable by their location within the cell, with PECT being a predominantly cytosolic enzyme, whereas the majority of PCCT is found in the particulate fraction, in a similar manner to rat liver (33). However, a significant proportion of PECT activity is also found in the particulate fraction, in contrast to rat liver in which the enzyme possesses an exclusively cytosolic location (33), suggesting that a proportion of PECT activity is associated with cellular structures in human brain.

Phosphocholine and phosphoethanolamine possess concentrations of approximately 350 μM and 250 μM, respectively, in autopsied human brain (2). These values are most likely similar to concentrations found *in vivo*, since phosphoethanolamine levels are stable post mortem (25). Referring to Table 3, it is evident that both enzymes are operating with phosphobase concentrations close to their K_m, implying that flux through this step of the pathway is moderately sensitive to the cellular concentrations of these compounds. We also observed that the K_m of phosphocholine for particulate PCCT was significantly higher than that for the cytosolic enzyme, and that this difference was likely not due to the absence of lipids in the cytosolic fraction. This suggests that the cytosolic enzyme could be distinct from that in the particulate fraction, possibly as a consequence of posttranslational modifications such as phosphorylation, as has been observed in other systems (34,35).

CTP was found to possess a millimolar K_m for human brain PECT and PCCT, in contrast to its concentration within the brain of only 100 μM (36). A similarly high K_m of CTP has been reported for PCCT activity in rat lung microsomes in which CTPase activity had been inhibited (1 mM) (40), rat lung cytosol (0.7–1.0 mM) (40), PCCT transfected s*f*9 cells (0.7 mM) (35), CHO cells (0.5 mM) (41), and rat brain (10 mM) (31). However, the K_m of CTP for human brain PECT (approximately 1.3 mM) is much higher than that reported for rat liver PECT activity (approximately 50 μM) (20,32). The high K_m/concentration ratio for CTP may underlie observations that cytidine supplementation increases the rate of phospholipid synthesis in cell culture and brain slice models (37,38), and suggests that the major mechanism by which CDPcholine elevates phospholipid levels in the brain is by increasing the availability of cytidine, rather than that of choline. This may well explain why, under some conditions, CDPcholine administration increases levels of brain PE in addition to that of PC (17,37), since CTP is common to both pathways. It should be noted that elevation of brain PE levels by CDPcholine is a smaller and less consistent effect of the drug, with Lopez-Coviella *et al.* (17) reporting increases under only some experimental conditions, and Agut *et al.* (39) observing unchanged abundance after 90 d of treatment.

In summary, we have derived the basic kinetic parameters of the two regulatory steps of phospholipid biosynthesis of human brain and have shown some differences in the characteristics of the pathways of PC and PE biosynthesis. This information will be useful in determining the mode of action of compounds designed to stimulate membrane synthesis, such as CDPcholine, as well as suggesting ways in which such drugs may be improved. Furthermore, since the activity of PECT, PCCT, ethanolamine kinase, and choline kinase is similar in autopsied and biopsied brain, it is likely that each enzyme is stable up to at least 12 h post mortem, thereby allowing examination of how the phospholipid biosynthetic pathway is affected by diseases of the brain.

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Isolation and Characterization of Inositol Sphingophospholipids from *Phytophthora parasitica* Dastur[1]

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ABSTRACT: Several inositol sphingophospholipids (ISPL) were isolated from mycelia of *Phytophthora parasitica* Dastur, a phytopathogenic fungus of carnation. The ISPL structures were determined by fast atom bombardment. All ISPL consisted of ceramides linked to inositol phosphate. We investigated the effect of growth conditions on the ISPL produced in four different media that are commonly used for fungal cultures. We showed that *P. parasitica* Dastur synthesized four major classes of compounds with molecular weights of M_r = 751, 807, 835, and 849 containing the 16:1 base and the 16:0 or 20:0 or 22:0 or 22h:1 *N*-acyl group. The relative abundance of the different ISPL is dependent on growth conditions.
Lipids 32, 359–362 (1997).

Inositol sphingophospholipids (ISPL) have been found in plants (1–5), yeasts (6–8), fungi (9), and protozoa (10–12), and all were shown to be derived from myoinositol phosphorylceramide (7,10). The ceramides typically contain C_{16}- or C_{18}-sphingosine or C_{18}-phytosphingosine (7,10) as long-chain base (LCB), which is acylated with palmitic, stearic (10, 11), 2- or 3-hydroxyhexacosanoic or 4-hydroxy-2-docosenoic acid (7) to form the backbone of the glycosphingophospholipids (13).

In a previous work, we isolated ISPL from mycelia of *Phytophthora capsici*, a phytopathogenic fungus of pepper (*Capsicum annuum* cv. Yolo Wonder) (14). The amide-linked fatty acids were found to have chains of 16, 20, and 22 carbon atoms. The LCB were identified as trimethylsilyl derivatives and shown to be C_{16}-sphingosine and C_{16}-dihydrosphingosine. We showed that *P. capsici* synthesized one major class of compounds with molecular weights of M_r = 833 and 835 containing the 16:1 base and the 22:1 or the 22:0 *N*-acyl group, independent of the growth medium used (15).

These ISPL induce a protective response by cotyledons of young peppers against necrotic lesions caused by the pathogen *P. capsici* (14,15).

In continuation of our work on ISPL of the *Phytophthora* genus, we determined whether ISPL were present in the strain 26 of *P. parasitica* Dastur, a phytopathogenic fungus of carnation and whether their composition was dependent on growth conditions.

MATERIALS AND METHODS

Microorganism and growth. The isolate 26 of *P. parasitica* Dastur from the fungal culture collection of INRA Antibes was grown on M1, M2, M3, and M4 media.

M1 was the Huguenin medium (16) which contained (g/L): glucose, 10; K_2HPO_4, 0.2; KH_2PO_4, 0.8; K_2SO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCO_3$, 0.2; KNO_3, 1; thiamine, 0.002; $FeSO_4 \cdot 7H_2O$, 0.0005; $ZnSO_4 \cdot 7H_2O$, 0.0005; $CuSO_4 \cdot 7H_2O$, 0.00002; $MnCl_2 \cdot 7H_2O$, 0.00002; $Mo_7O_{24}(NH_4)_2$, 0.00002; after sterilization M1 was supplemented with 10 mL of alcoholic solution which contained 2 mg/mL of cholesterol. M2 was the Hall medium (17) which contained (g/L): glucose, 30; L-asparagine, 10; K_2HPO_4, 0.26; KH_2PO_4, 0.47; $MgSO_4 \cdot 7H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.001; thiamine, 0.001; $CaCl_2 \cdot 2H_2O$, 0.01; $ZnSO_4 \cdot H_2O$, 0.001; $CuSO_4.5H_2O$, 0.0003; $Na_2Mo \cdot 2H_2O$, 0.0002; $MnCl_4 \cdot 4H_2O$, 0.0002; with pH adjusted to 6.5. M3 was the Plich and Rudnicki medium (18) which contained (g/L): glucose, 25; yeast extract, 5; L-asparagine, 1; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 1; thiamine, 0.001; with pH adjusted to 6. M4 was a chemically defined medium which contained (g/L): glucose, 20; L-asparagine, 1; KH_2PO_4, 0.6; $K_2HPO_4 \cdot 3H_2O$, 0.125; KNO_3, 0.7; $MgSO_4 \cdot 7H_2O$, 0.25; $Ca(NO_3)_2$, 0.3; $ZnSO_4 \cdot 7H_2O$, 0.004; $MnSO_4 \cdot H_2O$, 0.0015; H_3BO_3, 0.001; $Na_2MoO_4 \cdot 2H_2O$, 0.001; $CuSO_4 \cdot 5H_2O$, 0.00002; KI, 0.00002; $CaCl_2 \cdot 6H_2O$, 0.00002; $FeSO_4 \cdot 7\ H_2O$, 0.0055; sodium ethylenediaminetetraacetate, 0.0075; nicotinic acid, 0.001; pyridoxine, 0.001; calcium pantothenate, 0.001; thiamine, 0.001; with pH adjusted to 6.

Fungi were grown at 24°C under 16 h of light per day on M1, M2 and M4, and in the dark on M3 medium. Mycelia were collected after 3 wk of growth on M1 and M3 media, after 10 d of growth on M4 medium, and after 3 d on M2 medium.

Extraction and fractionation of lipids. Mycelial walls were isolated from fungi as described by Fabre *et al.* (19), and were treated with chloroform/methanol (2:1, vol/vol). The extracted

[1]This work is dedicated to the memory of Professor G. Michel.

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Abbreviations: GC, gas chromatography; FAB, fast atom bombardment; HPTLC, high-performance thin-layer chromatography; ISPL, inositol sphingophospholipid; LCB, long-chain base; MS, mass spectrometry.

lipids dissolved in a minimum volume of chloroform were separated into neutral and polar lipids by column chromatography on Bio-Sil HA silicic acid (BioRad, Ivry sur Seine, France) (20). Neutral lipids were eluted with chloroform, and polar lipids were eluted with chloroform/methanol (95:5, vol/vol; 90:10, vol/vol; 75:25, vol/vol; 50:50, vol/vol) and pure methanol. ISPL were eluted with chloroform/methanol (50:50, vol/vol).

Thin-layer chromatography of lipid fractions eluted from the silicic acid column. Lipid fractions were analyzed by high-performance thin-layer chromatography (HPTLC) on silica gel 60 plates (Merck; Darmstadt, Germany) that were developed with the solvent system described by Heape *et al.* (21). The detection of lipid fractions was carried out by spraying the plates with various reagents: 0.2% (wt/vol) vanillin in sulfuric acid/water (1:9, vol/vol), Dittmer and Lester reagent as modified by Vaskovsky and Kostetsky (22), Draggendorf reagent as modified by Wagner *et al.* (23). Identifications were made by comparison with authentic standards.

Analytical methods. Hydrolysis of phospholipids was performed with 1 M HCl for 16 h at 100°C or 6 M HCl for 48 h at 100°C. Hydrolysates were reduced and acetylated according to the method of Sawardeker *et al.* (24). Alditol acetates were analyzed by gas chromatography (GC) using Intersmat instrument (Model 120 FL; Intersmat, Lyon, France) fitted with an SP2380 glass capillary column (0.32 mm × 30 m). The column temperature was programmed from 140 to 260°C at 4°C/min.

Mass spectrometry (MS). Fast atom bombardment (FAB) mass spectra of phospholipids were recorded in the negative ion mode on a VG ZAB2 SEQ mass spectrometer (Manchester, United Kingdom). The instrument was equipped with a VG FAB standard source with a cesium ion gun delivering about 2 μA of cesium ion current at about 35 kV.

Spectra were obtained at a magnet scan rate of 20 s/decade over a mass range of *m/z* 200–2000 at 8 kV. The mass range was calibrated using cesium iodine clusters. The spectra were collected and processed using the VG II-250 J data system. Samples were dissolved in chloroform/methanol (1:1, vol/vol) and mixed with an equal volume of triethanolamine. The sample (2 μL) was deposited on the FAB probe tip mixed with matrix, i.e., glycerol.

RESULTS AND DISCUSSION

The percentages of total lipids extracted from the mycelial cell walls are given in Table 1. *Phytophthora parasitica*, strain 26, is characterized by a large total lipid content. The same percentage was observed when strain 26 was grown on M2, M3, and M4 media whereas this fungus is characterized by the lowest amount of lipids in the M1 medium.

Total lipids were fractionated on a column of Bio-Sil HA silicic acid to measure the relative amounts of neutral (eluted with chloroform) and polar (eluted with chloroform containing increasing concentrations of methanol) lipids as detailed in the Materials and Methods section (Table 1). The differences indicate that the relative abundance of neutral and polar lipids is dependent on growth conditions. The presence of L-asparagine in the medium or the time of growth of the fungus affected the percentage of polar lipids. The amount of polar lipids found in strain 26 grown on medium M3 was lower than in M1 medium without L-asparagine for the same time of growth. In the M4 medium the percentage of polar lipids was higher than in the M3 medium. M3 and M4 media contained the same amount of L-asparagine, but the time of growth was longer in M3 medium. The polar lipids consisted of phospholipids as revealed by HPTLC and spraying with reagent for phospholipids. HPTLC analysis of phospholipids eluted with chloroform/methanol (50:50, vol/vol) showed the presence of a major compound that comigrated with and had the molecular ion of, ISPL, as previously described (14,15). No other additional peaks were observed. This indicates that the ISPL was essentially pure, and that any other phospholipids eluting with it are present in very small amounts. No compound was revealed by specific reagent for tertiary amines. *Phytophthora parasitica* does not produce phosphatidylcholine. Previously, Pivot *et al.* (25) have shown that

TABLE 1
Lipid Accumulation in *Phytophthora parasitica* Dastur, Strain 26[a]

Culture medium	Total lipid content[b] (%, w/w)	Percentage of each lipid fraction[c] (%, w/w)	
		Neutral lipids	Polar lipids
M1	16.5	52.8	47.2
M2	32.0	55.0	45.0
M3	35.0	81.8	18.2
M4	35.0	28.5	71.5

[a]Strain 26 of *P. parasitica* Dastur was grown on M1, M2, M3, and M4 media. M1 was the Huguenin medium (Ref. 16); M2 was the Hall medium (Ref. 17); M3 was the Plich and Rudnicki medium (Ref. 18); M4 was a chemically defined medium. The compositions of M1, M2, M3, and M4 media and the conditions of cultures were described in the Materials and Methods section.
[b]These cultures were used to prepare the lipid extracts from fungal cell walls. The wt% of total lipids content was expressed as a percentage of the dry weight of fungal cell walls.
[c]The weight percentage of neutral and polar fractions eluted with chloroform and chloroform containing increasing concentrations of methanol and pure methanol, respectively, from Bio-Sil HA silicic acid chromatography (BioRad, Ivry sur Seine, France) was expressed as a percentage of total lipids extracted.

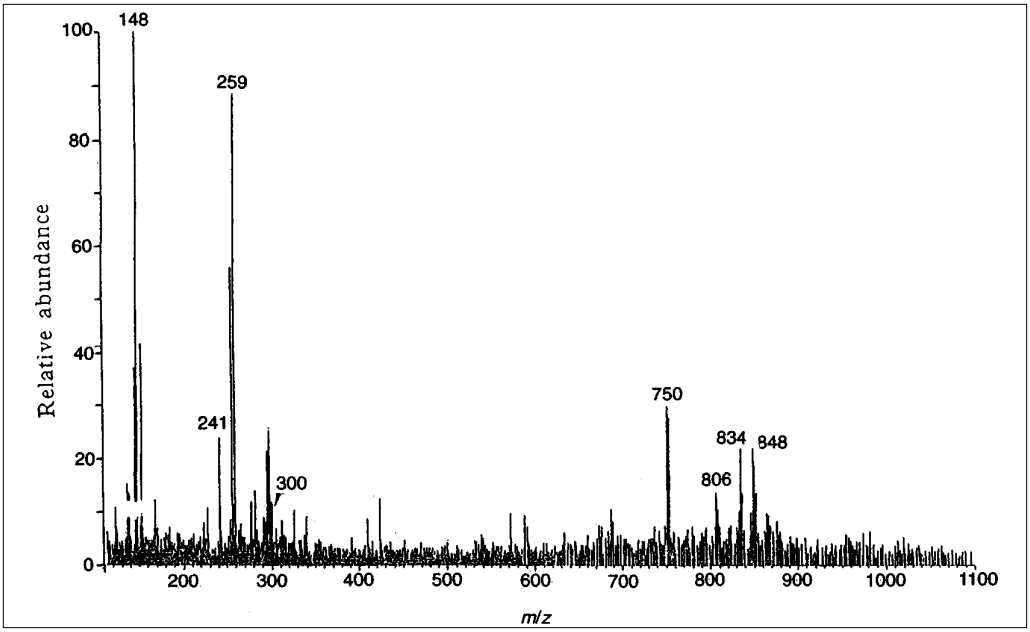

FIG. 1 . The negative fast atom bombardment mass spectrum of inositol sphingophospholipids from *Phytophthora parasitica* Dastur strain 26, grown on M2 medium. The ions at *m/z* 750, 806, 834, 848 correspond to individual molecular species present. Fragments in the spectrum at *m/z* 241, 259, and 300 are characteristic of an inositol sphingophospholipid as reported by Dommon and Costello (Ref. 27) and Ohashi (Ref. 29).

strain 107 of *P. capsici* grown on a chemically defined medium (26) produced dihexadecanoyl phosphatidylcholine.

The ISPL structure was determined by FAB MS. The negative ion FAB mass spectrum of the ISPL fraction from strain 26 grown on M1, M2, M3, and M4 media showed molecular species with $[M - H]^-$ ions at *m/z* 750, 752, 806, 808, 832, 834, 848, and 850. As an example, Figure 1 shows the mass spectrum of the ISPL fraction from strain 26 grown on M2 medium. This mass spectrum is similar to that of the ISPL previously described (15). It shows the characteristic ion corresponding to the cleavage of the inositol–phosphate bond [M − H − 162], and ions at *m/z* 241 and 259, the formation of which has been previously reported (11,27–29). The results indicate that in strain 26 of *P. parasitica* Dastur grown on M1, M2, M3, and M4 media the major ISPL correspond to the compounds of molecular weights 751, 807, 835, and 849. Although the same ISPL are present in the strain 26 grown on all media, there are significant differences in their relative abundance depending on growth conditions. This is especially true in regard to the ISPL corresponding to the compounds of molecular weights 751, 835, and 849. The ISPL, $M_r = 751$ is a major compound when strain 26 is grown on Huguenin medium (M1) and on Hall medium (M2). The ISPL with $M_r = 835$ and $M_r = 849$ were observed to be the most abundant compounds found in strain 26 grown on Plich and Rudnicki medium. Strain 26 grown on synthetic medium (M4) produced the four molecular species in similar percentage. Earlier studies have shown that *P. capsici*, strains 197, 107, and 375 synthesize only one major class of compounds with molecular weights of $M_r = 833$ and 835. It seems that *P. parasitica* is different and is characterized by the presence of four major molecular species, the amounts of which are affected by growth conditions.

The data from tandem MS, reported earlier (15,27), suggested that these different ISPL were compounds mainly of a C_{16}-dihydrosphingosine LCB linked *via* a hexacosanoyl (16:0) or eicosanoyl (20:0) or docosanoyl (22:0) or hydroxydocosenoyl (22:1) amide bond (Fig. 2).

ISPL produced by *P. parasitica* Dastur are not glycosylated. Hydrolysis of ISPL fraction with 1 M or 6 M HCl at 100°C for 16 h or 48 h gave only inositol, identified by GC of the acetyl derivative. No sugar was found in the hydrolysate. They are similar to ISPL isolated from *P. capsici* (15). This structure seems characteristic of the genus *Phytophthora*. It is similar to ISPL from *Saccharomyces cerevisiae* (7) and *Leishmania donovani* (10).

The biological activity of the sphingophospholipids had already been previously demonstrated (14,15). They induce a

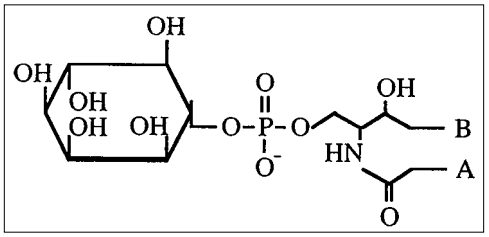

FIG. 2 . Structures of major molecular species of inositol sphingophospholipids of molecular weights 751 (B = -CH=CH-$(CH_2)_{10}$-CH_3; A = -$(CH_2)_{14}$-CH_3), 807 (B= -CH=CH-$(CH_2)_{10}$-CH_3; A = -$(CH_2)_{18}$-CH_3), 835 (B= -CH=CH-$(CH_2)_{10}$-CH_3; A = -$(CH_2)_{20}$-CH_3) and 849 (B= -CH=CH-$(CH_2)_{10}$-CH_3; A = -CH=CH-CH(OH)-$(CH_2)_{17}$-CH_3).

protective response by cotyledons of young peppers against necrotic lesions caused by the pathogen *P. capsici*. They are good models for studies in host–parasite interactions.

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Nuclear Magnetic Resonance Characterization of 6α-Chloro-5β-cholestane-3β,5-diol Formed from the Reaction of Hypochlorous Acid with Cholesterol

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ABSTRACT: Hypochlorous acid generated by neutrophil myeloperoxidase has been shown to convert cholesterol into three different chlorohydrin isomers which previously had not been fully characterized. We have reacted hypochlorous acid with cholesterol/1,2-dipalmitoyl phosphatidylcholine liposomes to give these three major products and established that they are 6β-chloro-5α-cholestane-3β,5-diol (chlorohydrin **1**), 5α-chloro-6β-cholestane-3β,6-diol (chlorohydrin **2**) and 6α-chloro-5β-cholestane-3β,5-diol (chlorohydrin **3**). These products were separated by thin-layer chromatography and fully characterized by ^{1}H, ^{13}C, attached proton test, doublequantum correlation spectroscopy, total correlation spectroscopy, heteronuclear multiple bond correlation and heteronuclear multiple quantum coherence nuclear magnetic resonance spectroscopy.
Lipids 32, 363–367 (1997).

Hypochlorous acid is a strong oxidant generated from H_2O_2 and Cl^- by the myeloperoxidase system of neutrophils and monocytes (1). It reacts readily with a wide range of biological molecules including thiols, thioethers, iron–sulfur centers, heme groups, and amines (2–4). As such, HOCl is a likely contributor to tissue injury in inflammation. HOCl is known to form chlorohydrin addition products with unsaturated fatty acids and cholesterol when they are exposed as isolated lipids (5–8), red cell membranes (8), and possibly lipoproteins (9). The pathophysiological significance of these products is not known.

Three major products have been identified from cholesterol liposomes treated with reagent or myeloperoxidase-derived HOCl (Fig. 1). Two have been characterized by mass spectrometry (MS) and comparison with synthesized standards (7,8) as the chlorohydrin isomers **1** and **2**. The third product has been shown by MS to be a chlorohydrin (7,8), but until now has not been fully characterized. In this paper we have used one-dimensional and two-dimensional nuclear magnetic resonance (NMR) spectroscopy to show that this product is 6α-chloro-5β-cholestane-3β,5-diol **3**, a product that has previously been found after chlorine bleaching of wood pulp (10,11). A similar assignment was also carried out on **1** and **2** for comparison.

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Abbreviations: APT, attached proton test; DPPC, 1,2-dipalmitoyl phosphatidylcholine; DQCOSY, doublequantum correlation spectroscopy; GC–MS, gas chromatography–mass spectrometry; HMBC, heteronuclear multiple bond correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence spectroscopy; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; TLC, thin-layer chromatography; TOCSY, total correlation spectroscopy.

EXPERIMENTAL PROCEDURES

Materials. Cholest-5-en-3β-ol, 5,6α-epoxy-5α-cholestan-3β-ol (epoxide **4**), 5,6β-epoxy-5β-cholestan-3β-ol (epoxide **5**), and 1,2-dipalmitoyl phosphatidylcholine (DPPC) were obtained from Sigma Chemical Co. (St. Louis, MO). Reference samples of 6β-chloro-5α-cholestane-3β,5-diol (chlorohydrin **1**) and 5α-chloro-6β-cholestane-3β,6-diol (chlorohydrin **2**) were synthesized from cholesterol epoxides **4** and **5**, as previously described (8). Other biochemicals and high-performance liquid chromatography grade solvents were obtained from BDH Laboratory Supplies (Poole, England). Silica gel thin-layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Germany). Sodium hypochlorite was obtained from Reckitt and Colman (Auckland, New Zealand). This was diluted in phosphate-buffered saline (PBS) and brought to physiological pH with aqueous HCl before standardizing by reacting with 5-thiobis(2-nitrobenzoic acid) and measuring the change in A_{412} (ε = 14100 M^{-1} cm^{-1}) (12).

Treatment of cholesterol with HOCl. Cholesterol/DPPC (1:1) liposomes were prepared in PBS and treated with HOCl as described previously (8). After 30 min at room temperature, the solution was acidified with a small amount of aqueous HCl and extracted twice with two times the volume of dichloromethane. Purification by TLC gave the chlorohydrins **1**, **2** (28% combined), **3** (24%), and 29% of unreacted cholesterol (7,8,13). The chlorohydrin **3** was also prepared by adding a tenfold excess of HOCl to a solution of cholesterol

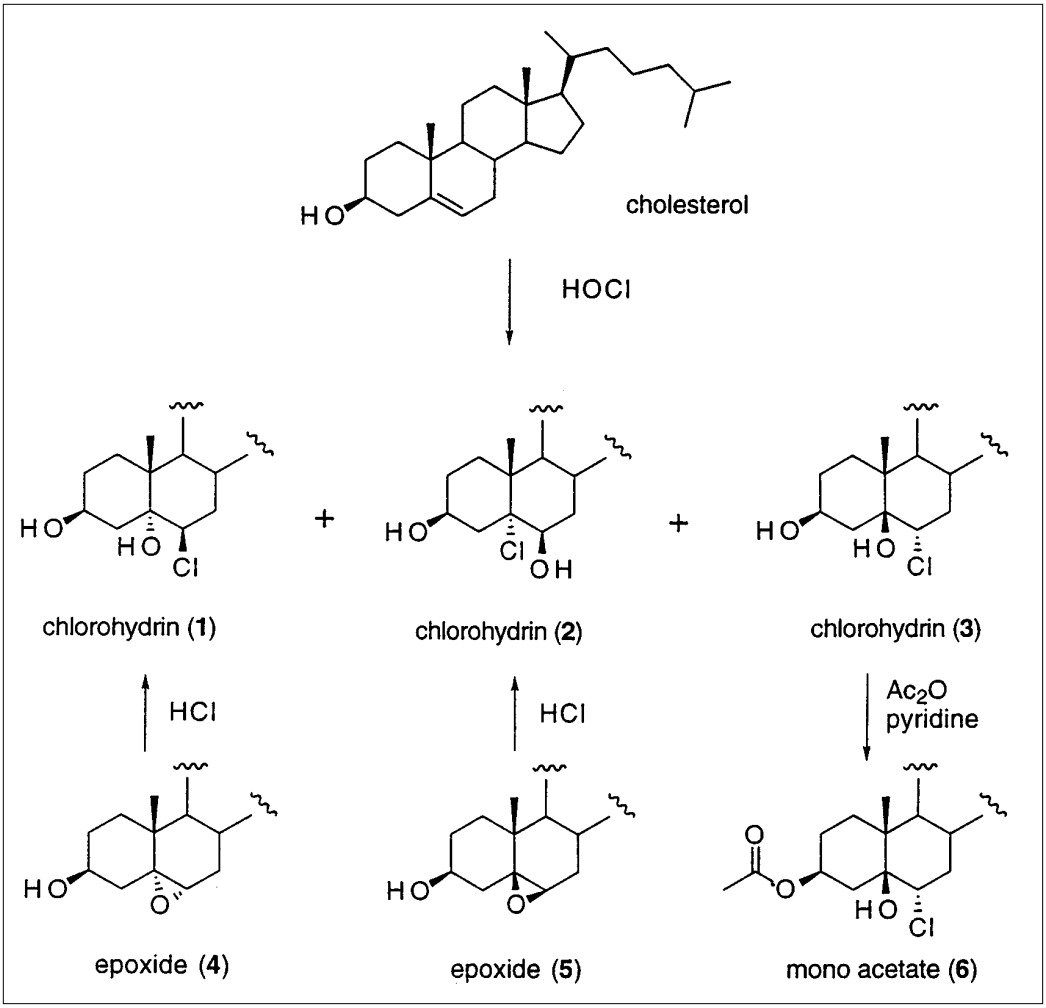

FIG. 1. Preparation of compounds **1–3** and **6.**

in acetonitrile/isopropanol (1:1) at room temperature. An equal volume of water was added after 30 min, and the mixture was extracted twice with dichloromethane. TLC gave the chlorohydrin **3** (30% of starting material) (10,11). The products were characterized by electrospray ionization MS as described previously (8,14).

Sample derivatization. The monoacetate **6** (3β-acetoxy-6α-chloro-cholestan-5β-ol) was prepared by dissolving **3** in pyridine containing an excess of acetic anhydride and reacting this overnight at room temperature (see Fig. 1). The solution was diluted with dichloromethane and washed sequentially with 5% aqueous HCl, 5% aqueous $NaHCO_3$, and water before drying with $MgSO_4$. The final solution was filtered and concentrated under N_2 for NMR analysis.

NMR. NMR spectra were recorded in $CDCl_3$ solution on Varian XL-300 and Varian UNITY 300 spectrometers (Palo Alto, CA) equipped with a Varian 5-mm broad band probe and a Nalorac Z-SPEC 3-mm inverse detection probe (Varian), respectively, and operating at 300 and 75 MHz for ^1H and ^{13}C, respectively. Chemical shifts are reported in ppm relative to tetramethylsilane. Doublequantum correlation spectroscopy (DQCOSY), total correlation spectroscopy (TOCSY), attached proton test (APT), heteronuclear multiple bond correlation spectroscopy (HMBC), and heteronuclear multiple quantum coherence spectroscopy (HMQC) experiments were recorded using standard Varian pulse sequences.

RESULTS

The structure and configuration of the chlorohydrin **3** were determined by ^1H NMR, ^{13}C NMR, ^1H-^1H COSY, TOCSY, APT, HMQC, and HMBC experiments. An assignment of the ^1H and ^{13}C NMR chemical shifts is given in Tables 1 and 2 with atom numbering shown in Figure 3. The ^1H NMR spectrum of the chlorohydrin **3** (Table 1 and Fig. 2) gave diagnostic resonances for the methyl groups at 0.65 ppm (singlet due to H_{18}), 0.84/0.85 ppm (two doublets due to H_{26} and H_{27}), 0.91 ppm (doublet due to H_{21}) and 0.99 ppm (singlet due to H_{19}). Other chemically distinct and diagnostic resonances were observed at 3.30 ppm (broad, D_2O exchangeable, C_3- and C_5-OH), 4.18 ppm (broad singlet, H_3) and 4.30 ppm (*dd,* J = 4.9, 12.7 Hz, H_6). By comparison, H_3 appears as a com-

TABLE 1
Selected ^{1}H NMR Data for 3

Proton number	Chemical shift (ppm)
H_1	1.84, 1.33
H_2	1.60
H_3	4.18
C_3-OH	3.12
H_4	2.12, 1.90
C_5-OH	3.30
H_6	4.30 (*dd J* = 4.9, 12.7 Hz)
H_7	2.05, 1.35
H_9	1.30
H_{12}	1.95, 1.10
H_{18}	0.65 (*s*)
H_{19}	0.99 (*s*)
H_{20}	1.36
H_{21}	0.91 (*d J* = 6.5 Hz)
H_{22}	1.37, 0.98
$H_{26/27}$	0.84, 0.85 (2 × *d J* = 6.4 Hz)

plex multiplet for the alternative *trans*-fused (5α,10β) chlorohydrin **1** (4.03 ppm) and the chlorohydrin **2** (4.32 ppm). It should also be noted that the H_{19} methyl groups of **1** (1.27 ppm) and **2** (1.27 ppm) were observed in a significantly downfield position relative to those of **3** (0.99 ppm).

TABLE 2
^{13}C Chemical Shifts for 1, 2, and 3

Carbon number	Chemical shift (ppm)		
	1	**2**	**3**
C_1	32.96	33.66	25.72
C_2	30.70	30.49	27.55
C_3	67.68	67.86	67.24
C_4	41.88	41.39	31.45
C_5	76.90	84.23	77.12
C_6	63.85	75.75	67.77
C_7	36.14	34.08	38.84
C_8	30.12	30.42	35.70[a]
C_9	45.75	46.09	42.81
C_{10}	39.04	39.52[a]	42.70[b]
C_{11}	21.16	21.26	21.43
C_{12}	39.86	39.78	39.72
C_{13}	42.73	42.77	42.78[b]
C_{14}	55.40	55.77	56.09[c]
C_{15}	24.09[b]	24.09[b]	24.05[b]
C_{16}	28.17	28.21	28.13
C_{17}	56.22	56.17	56.09[c]
C_{18}	12.15	12.19	11.97
C_{19}	18.38	18.27	16.73
C_{20}	35.76	35.79	35.70[a]
C_{21}	18.64	18.67	18.61
C_{22}	35.51	36.16	36.09
C_{23}	23.84[b]	23.82[b]	23.80[b]
C_{24}	39.48	39.52[a]	39.45
C_{25}	27.99	28.01	28.00
C_{26}	22.54	22.56	22.55
C_{27}	22.81	22.81	22.80

[a]Overlapping resonances. [b]Tentative assignments. [c]Overlapping resonances.

Acetylation of **3**, using an excess of acetic anhydride in pyridine, gave a monoacetate **6** in which the H_3 resonance was characteristically shifted downfield to 5.38 ppm (11). A single acetate methyl resonance was also observed at 2.10 ppm for **6**. These results are consistent with **3** containing a single secondary alcohol at C_3, a tertiary alcohol at C_5, and a chlorosubstituent at C_6.

The assignment of the ^{1}H and ^{13}C NMR data of **3** (Tables 1 and 2) was undertaken, using literature data and the information detailed below, to confirm the A and B ring configurations and the substitution pattern as shown in Figure 3. A number of key proton connectivities were determined by DQCOSY spectroscopy. In particular, strong correlations were observed between 4.30 ppm (H_6) and both 1.35 ppm (H_7) and 2.05 ppm (H_7). That the resonances at 1.35 and 2.05 ppm were due to a methylene group was confirmed by a strong, mutual DQCOSY correlation and also by HMQC data (see later). The resonance due to H_3 at 4.18 ppm gave strong correlations to resonances at 2.12 ppm (H_4), 1.90 ppm (H_4), and 1.60 ppm H_2. The methylene assignments were established as above by HMQC (see later), and the observation of a strong DQCOSY correlation between the resonances at 2.12 and 1.90 ppm (H_4 methylene group). Numerous other correlations were evident in the DQCOSY spectrum of **3**, but these were difficult to assign unambiguously due to overlapping resonances. The sequences from H_2 through to H_4 and H_6 through to H_7 were confirmed by two-dimensional TOCSY correlations.

The literature on ^{13}C NMR data of steroids has been extensively reviewed (15) such that the base values for the chemical shifts of all carbons are clearly indicated. In addition, extensive sets of substituent effects are available to aid the assignment of new systems. An APT experiment established the methine resonances at 28.00, 35.70, 42.81, 56.09, 67.24, and 67.77 ppm and the methyl resonances at 11.97 (C_{18}), 16.73 (C_{19}), 18.61 (C_{21}), and 22.55, 22.80 ($C_{26/27}$) ppm (Table 2). The assignment of the remaining quaternary and methyl resonances was then possible.

An HMBC experiment provided a key starting point for the complete assignment of the ^{13}C resonances of **3** (see Fig. 3 and Table 2). In particular, the C_{18} methyl group protons correlated with resonances at 56.09 (C_{14} and C_{17}, coincident), 42.78 (C_{13}), and 39.72 ppm (C_{12}). The resonance due to the C_{19} methyl group at 0.99 ppm gave characteristic correlations to resonances at 77.12 (C_5), 42.70 (C_{10}), 42.81 (C_9), and 25.72 ppm (C_1). The resonance due to the C_{21} methyl group at 0.91 ppm correlated with resonances at 56.09 (C_{17}) and 35.70/36.09 ppm (C_{20} and C_{22}). The resonances for the C_{26} and C_{27} methyl groups gave characteristic correlations to 39.45 (C_{24}), 28.00 (C_{25}), and 22.55/22.80 ppm ($C_{26/27}$). Diagnostic long-range correlations were also evident for C_5-OH at 3.30 to 77.12 (C_5), 67.77 (C_6), and 42.70/42.78 ppm (C_{10}). Similarly, the resonance due to H_6 at 4.30 ppm gave correlations to 77.12 (C_5) and 31.45 ppm (C_4).

An HMQC experiment enabled the assignment of a number of other ^{13}C resonances of **3** (Table 2). In particular, the

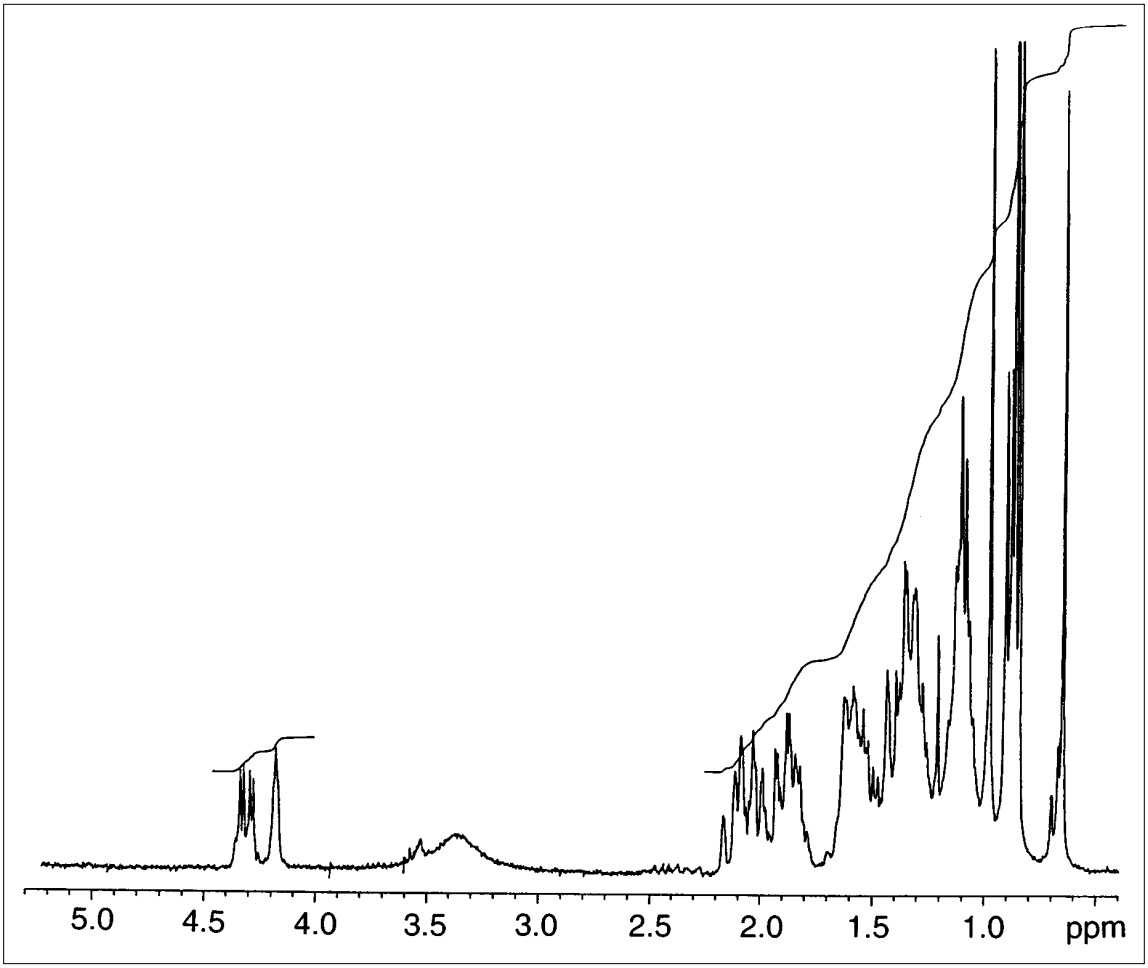

FIG. 2. ^{1}H nuclear magnetic resonance spectrum of **3**.

methylene proton resonances for H_1, H_2, H_4, H_7, H_{12}, and H_{22} correlated to ^{13}C resonances at 25.72, 27.55, 31.45, 38.84, 39.72, and 35.70 ppm, respectively. Other key correlations were evident between H_3 (4.18 ppm), H_6 (4.30 ppm), H_9 (1.30 ppm), H_{18} (0.65 ppm), H_{19} (0.99 ppm), H_{20} (1.36 ppm), H_{21} (0.91 ppm), and $H_{26/27}$ (0.84 and 0.85 ppm) and ^{13}C resonances at 67.24, 67.77, 42.81, 11.97, 16.73, 35.70, 18.61, and 22.55/22.80 ppm, respectively. The remaining ^{13}C NMR resonances were assigned by comparison with tabulated literature reference values.

A similar analysis was carried out on the alternative *trans*-fused (5α,10β) chlorohydrins **1** and **2** (Table 2). Again, APT and HMBC data provided the key to the assignment of the ^{13}C NMR data. A comparison of the ^{13}C NMR data obtained for **1**, **2**, and **3** reveals that variations in chemical shifts are limited to the A and B ring carbons, little change is evident for C_{11}–C_{18} and C_{20}–C_{27} resonances (Table 2). Of particular note is the effect, at C_5 and C_6, of a chloro vs. a hydroxyl substituent (compare the 5-OH, 6-Cl isomers **1** and **3** with the 5-Cl and 6-OH isomer **2**). The influence of a 5β-configuration (**3**) relative to a 5α-configuration (**1** and **2**) is most pronounced at C_1, C_2, C_4, C_6–C_{10}, and C_{19}.

DISCUSSION

Recent work by us (8) and others (7,13) has shown that the reaction of HOCl with cholesterol liposomes forms not only the chlorohydrins **1** and **2** but also another chlorohydrin isomer (**3**) which, until now, had not been fully characterized. Previous evidence for this product being a chlorohydrin was based on analysis by electrospray MS after TLC separation (8). Using ^{1}H and ^{13}C NMR techniques, we have been able to identify this product as 6α-chloro-5β-cholestane-3β,5-diol **3**. It is of interest to note that **3** had previously been reported by Lindgren (10,11) as resulting from the bleaching of wood components by chlorine. We have extended these original studies, using modern spectral techniques, in view of the possible pathophysiological significance of cholesterol chlorohydrin formation.

The configuration of **3** was assigned as shown in Figure 3, based on the partial assignment of the ^{1}H and ^{13}C spectral data of **3** summarized in Tables 1 and 2. The H_3 proton is axial in *trans*-fused (5α,10β)-steroids (e.g., the chlorohydrins **1** and **2** and the epoxide **4**) and appears as a complex multiplet at 4.03, 4.32, and 3.95 ppm, respectively. By comparison, the

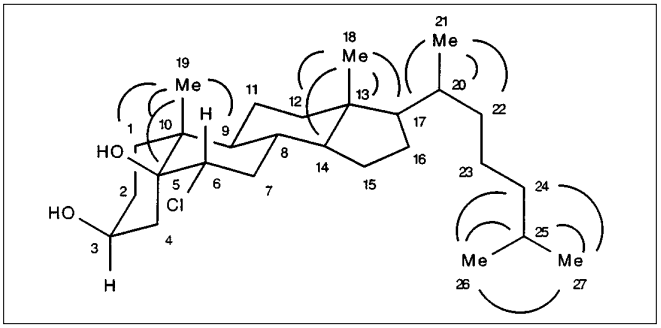

FIG. 3. Molecular structure of chlorohydrin **3** showing key heteronuclear multiple bond correlation spectroscopy correlations.

H_3 resonances of the *cis*-fused (5β,10β)-steroids, **3** and **5**, show less defined vicinal coupling to H_2 and H_4 such that H_3 is observed as a broad resonance at 4.18 and 3.70 ppm, respectively. The upfield position of the resonance for H_{19} in **3** (0.99 ppm), relative to **1** (1.27 ppm) and **2** (1.27 ppm), is also consistent with the proposed structure. Further support for the proposed configuration of **3** (Fig. 3) came from the observation of a nuclear overhauser enhancement between H_6 and H_{19}. The chlorohydrin **3** also gave a characteristic OH stretch at 3468 cm^{-1} in its infrared spectrum.

A number of 6-chloro *cis*-fused withanolides, bearing the same (5β,6α,10β)-configuration as **3**, have been previously characterized by X-ray crystallography and ^1H and ^{13}C NMR spectroscopy. Significantly, the chemical shift of C_6 in **3** (67.77 ppm, Table 2) is consistent with a 6β-chloro substituent as reported for the related withanolides [C_6 = 63.8–65.5 ppm (16,17)]. The chemical shift and coupling pattern observed for the H_6 resonance of **3** (doublet of doublets at 4.30 ppm, J = 4.9, 12.7 Hz, Table 2) and the literature 6-chloro withanolides [doublet of doublets at 4.40 ppm, J = 6, 10.5 Hz (16,17)] are also particularly diagnostic of a (5β,6α,10β)-configuration. The corresponding H_6 resonances of the *trans*-fused (5α,6β,10β) and chlorohydrins **1** and **2** show little or no coupling to the two H_7 protons.

The chlorohydrin **3** is a major product when cell membrane lipids are treated with HOCl or the myeloperoxidase/H_2O_2/Cl^- system (8). The increased stability and the decreased planarity of this chlorohydrin (Fig. 3), relative to the chlorohydrins **1** and **2**, may prove to be more disruptive to membranes. Further investigation of its biological properties is needed to establish whether it and related chlorohydrins are involved in neutrophil-mediated injury. It is known that the incorporation of small amounts of oxysterols into cell membranes can cause cytotoxicity (18). Similar cytotoxic effects may be expected for cholesterol chlorohydrins and these are currently being investigated.

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Separation and Quantitation of Linoleic Acid Oxidation Products in Mammary Gland Tissue from Mice Fed Low- and High-Fat Diets

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ABSTRACT: We have developed an assay for the isolation and quantitation by gas chromatography–mass spectrometry (GC–MS) of free 9- and 13-hydroxyoctadecadienoic acid (9-HODE, 13-HODE) in the mammary glands of female mice. Internal standards consisting of $^{18}O_2$-labeled analogs of 9- and 13-HODE are added to pulverized frozen tissue prior to extraction with ethanol. Nonlipid materials are removed in a chloroform/methanol/water extraction step. The remaining lipid material is methylated with ethereal diazomethane, and much of the nonoxygenated fatty acid methyl esters are removed *via* silica solid-phase extraction. Samples are either further derivatized with bis(trimethylsilyl)trifluoroacetamide to form the trimethylsilyl ethers for quantitative analysis by GC–MS or are analyzed as the methyl esters by chiral high-performance liquid chromatography to determine the enantiomeric distribution of the 9- and 13-HODE. The extraction and quantitation protocol was applied to the analysis of mammary glands for free 9- and 13-HODE from mice fed isocaloric diets containing 20% corn oil, 5% corn oil, or 20% beef tallow. Chiral analysis of the products showed higher production of 13(*S*)-HODE relative to 13(*R*)-HODE; the enantiomeric excess is most likely due to enzymatic production of 13-HODE superimposed on a background of autoxidative production of 13(*R*)- plus 9(*S*)- and 9(*R*)-HODE. In addition, the effect of sample handling and storage conditions on the formation of 9- and 13-HODE in the samples was assessed by exposing aliquots of a common pool of rat mammary gland tissue to specified conditions prior to analysis. This methodology will be important during investigations of the contribution of linoleate oxidation products to the enhancement of mammary tumorigenesis by dietary fat.
Lipids 32, 369–375 (1997).

Numerous international population correlation studies report a strong positive linear relationship between dietary fat consumption and human breast cancer incidence and/or mortality. The amount and/or type of dietary fat also has a profound effect on the development of mammary gland tumors in mice and rats. Increased amounts of ingested fat, in particular fats rich in the polyunsaturated fatty acid, linoleic acid, have been reported to increase the development of mammary gland tumors in an array of mouse and rat experimental animal models (1). In addition, the amount and/or type of dietary fat substantially influences growth of human breast carcinomas maintained (transplanted) in immune-deficient mice (2). While it is clear that the amount and/or type of dietary fat can enhance mammary gland tumorigenesis in experimental animals and can stimulate the growth of human breast carcinomas maintained in immune-deficient mice, the mechanism by which this dietary constituent stimulates these tumorigenic processes is not known.

Although a number of mechanisms have been reported to explain the stimulatory effect of high levels of dietary fat to mammary tumorigenic processes, to date, none of these mechanisms has proven to be entirely satisfactory. In this communication, we provide evidence that oxygenated metabolites (products) of linoleic acid, formed *via* free radical and/or enzymatic processes, are elevated in the mammary gland of mice consuming high-fat diets. We propose that these oxygenated products may contribute to the stimulation of mammary tumorigenesis associated with fat hyperalimentation.

The oxygenated products of primary interest to us are 9- and 13-hydroxyoctadecadienoic acid (9-HODE, 13-HODE) as these products have been shown to have a growth stimulatory effect in experimental animals in an array of organ sites including the mammary gland (1,3–6).

Numerous reports describe the isolation of hydroxylated polyunsaturated fatty acids, but none of the methods is directly applicable to the isolation of these compounds from mammary gland tissue, since the tissue contains an unusually high concentration of fat (7). Therefore, we report on the development of an effective methodology for the quantitation of 9- and 13-HODE in the mammary glands of mice fed isocaloric diets containing different types and/or amounts of fat.

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Abbreviations: ANOVA, analysis of variance; BHT, butylated hydroxytoluene; BSTFA, bis(trimethylsilyl)trifluoroacetamide; GC–MS, gas chromatography–mass spectrometry; 9-HODE, 9-hydroxyoctadecadienoic acid; 13-HODE, 13-hydroxyoctadecadienoic acid; HPLC, high-performance liquid chromatography; ME, methyl ester; TMCS, trimethylchlorosilane; TMS, trimethylsilyl.

EXPERIMENTAL PROCEDURES

Chemicals and materials. Bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (BSTFA + 1% TMCS) was purchased from Supelco, Inc. (Bellefonte, PA). Diazomethane was prepared from Diazald® purchased from Aldrich (Milwaukee, WI). Butyrylcholinesterase was purchased from Sigma Chemical Company (St. Louis, MO). Oxygen-18 labeled water (97.4 atom %^{18}O) was from Isotec, Inc. (Miamisburg, OH). 13(*R,S*)-hydroxy-(*Z,E*)-9,11-octadecadienoic acid, 13(*S*)-hydroxy-(*Z,E*)-9,11-octadecadienoic acid, 9(*R,S*)-hydroxy-(*E,Z*)-10,12-octadecadienoic acid, and 9(*S*)-hydroxy-(*E,Z*)-10,12-octadecadienoic acid were purchased from Cayman Chemical Company (Ann Arbor, MI). Silica solid-phase extraction columns were purchased from Baxter Health Care Corporation (McGaw Park, IL). The Anspec Company, Inc. was the supplier for the J&W DB5-MS capillary GC column (Folsom, CA). Reverse-phase high-performance liquid chromatography (HPLC) was performed on a μBondapak C18 column from Waters (Milford, MA). Chiral HPLC was performed on a Chiralcel OD column from Chiral Technologies, Inc. (Exton, PA).

Preparation of internal standards. The preparation of $^{18}O_2$-labeled 9- and 13-HODE utilized the procedure of cholinesterase-catalyzed oxygen exchange (8,9). Oxygen-18 labeled HODE were prepared by the addition of 50 μg of 9- or 13-HODE dissolved in 20 μL methanol to 500 μL $H_2{}^{18}O$ containing 30 units of butyrylcholine esterase. After incubation for 24 h at 37°C, the reaction mixture was extracted four times with ethyl acetate, combined, dried under nitrogen, and redissolved in ethanol. The concentration of the solution was determined by measuring the absorbance at 234 nm and using a value of 23000 (10) mol L^{-1} cm^{-1} for the molar absorptivity constant (ε). Standards were stored at –20°C.

Diet study. Twenty-four female Balb/c mice (Harlan Sprague-Dawley, Indianapolis, IN) were divided into three groups at three weeks of age. Each group (eight mice/group) was placed on one of three chemically purified isocaloric diets differing only in the amount or type of fat. The mice were fed either a high-saturated-fat diet (20%, by weight, beef tallow), a low-unsaturated-fat diet (5%, by weight, corn oil), or a high-unsaturated-fat diet (20%, by weight, corn oil) for five weeks. The composition of these diets was as previously described (11). At the end of the five-week period, mice from each of the diet groups were sacrificed on the day the glands were to be extracted, and three mammary gland pairs were excised. The mammary glands excised from each mouse were processed and analyzed separately. The excised tissue was immediately placed in liquid nitrogen. All of the glands from each mouse were pulverized in liquid nitrogen and divided into two approximately equal samples: one for quantitation by gas chromatography–mass spectrometry (GC–MS), the other for chiral analysis.

Sample preparation and derivatization. A protocol for sample preparation including isolation and derivatization of 9- and 13-HODE from mammary gland tissue is shown in Figure 1. The 50-μL aliquots of $^{18}O_2$-labeled 9- and 13-HODE (18.8 and 29.1 ng/μL, respectively) were added to the pulverized tissue prior to extraction with ethanol for those samples analyzed by GC–MS. The samples for chiral analysis received no internal standard.

The mammary gland sample (100–200 mg) was pulverized in liquid nitrogen prior to two extractions in ethanol (10 and 5 mL). These extracts were combined and centrifuged in a refrigerated room (10°C). The supernatant was transferred to a pear-shaped flask, and the ethanol was evaporated *in vacuo*. Nonlipid material was removed (12) from the residue by extraction with dichloromethane/methanol/water (4:2:1.5, by vol). [Dichloromethane was used here because it has been reported that dichloromethane is less likely to promote autoxi-

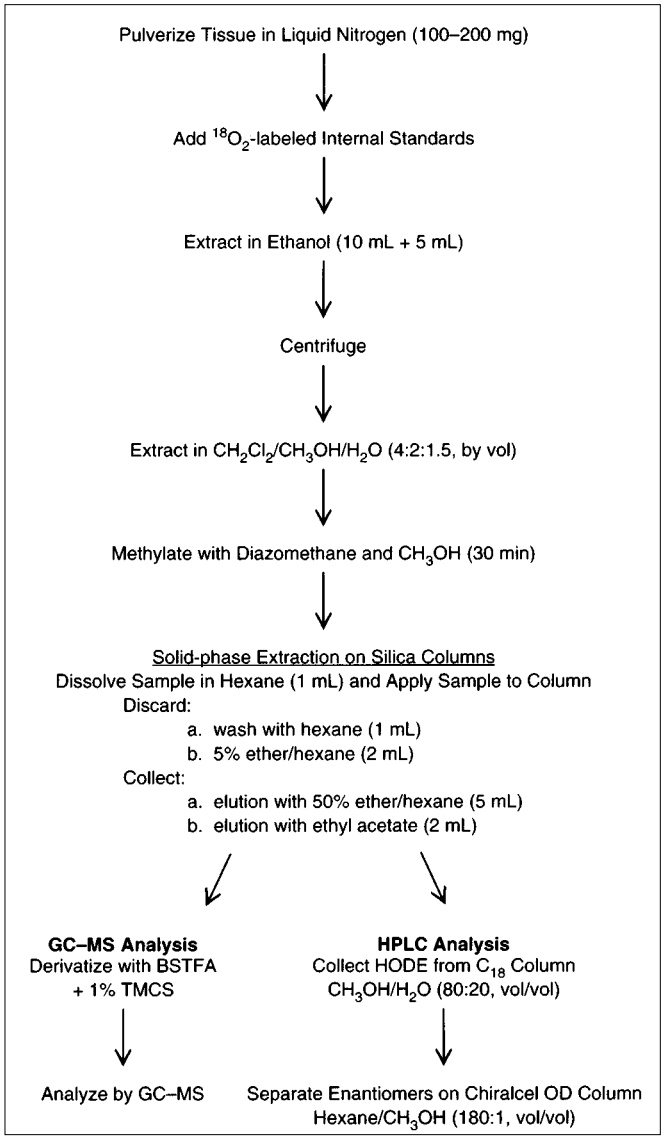

FIG. 1. Extraction procedure for isolating and derivatizing hydroxylated fatty acids from mammary gland tissue. GC–MS, gas chromatography–mass spectrometry; BSTFA, bis(trimethylsilyl)trifluoroacetamide; TMCS, trimethylchlorosilane; HPLC, high-performance liquid chromatography; HODE, hydroxyoctadecadienoic acid.

dation of the sample (13,14)]. The mixture of dichloromethane/methanol/water separated into two phases; the upper phase was pipetted off and discarded. The lower phase was evaporated *in vacuo*, and the residue was methylated with ethereal diazomethane and methanol. After 30 min, the reaction mixture was evaporated under nitrogen, and the residue was redissolved in 1 mL hexane. The esterified extract was applied to a 200-mg silica solid-phase extraction column that had been preconditioned with 2 mL hexane, 2 mL 50% ether/hexane, and 2 mL hexane. Following application of the samples to the columns, the nonpolar (nonhydroxylated) esters were eluted from the column with 2 mL 5% ether/hexane and discarded. Methyl esters (ME) of the hydroxylated analytes were eluted and collected with 5 mL 50% ether/hexane (15) followed by 2 mL ethyl acetate, then dried *in vacuo*. Those sample extracts (fraction containing hydroxylated esters) for analysis by GC–MS were derivatized with 50 µL BSTFA + 1% TMCS in pyridine at 60°C for 30 min; sample extracts to be analyzed by HPLC were redissolved in hexane.

Detection by GC–MS. Analysis of the mammary gland extract by GC–MS was performed on a Varian Saturn II ion trap mass spectrometer (Walnut Creek, CA). Samples were chromatographed on a J&W DB5-MS 30 m × 0.25 mm i.d. column with a 25-µm film thickness in a Varian 3400 gas chromatograph. The GC temperature program was 100°C (2-min hold) to 160°C at 10°C/min, 2°C/min to 225°C, and 40°C/min to 300°C (hold for 5 min). Peak areas under profiles at selected *m/z* values (*m/z* 382 for the analyte, *m/z* 386 for the internal standard: ^{18}O-labeled 9- and 13-HODE) at designated times were used for quantitation. The concentration of the 9- and 13-HODE in the extracted sample was calculated by Equation 1 (16):

$$\frac{\text{area } (m/z\ 382)}{\text{area } (m/z\ 386)} \times \frac{\text{amount of internal standard added}}{\text{mg tissue}} = \frac{\text{amount HODE}}{\text{mg tissue}} \quad [1]$$

HPLC analyses. The HPLC system used for these analyses was constructed from the following components: a Waters Corporation (Milford, MA) 6000A pump, a Rheodyne (Cotati, CA) 7125 manual injector with a 5-µL sample loop, and a Kratos Analytical (Anspec, Ann Arbor, MI) Spectroflow 757 absorbance detector set to a wavelength of 234 nm. Data were collected and processed with a Waters Corporation Millennium Data System.

Prior to chiral analysis, 9- and 13-HODE ME were collected from a reverse-phase separation on a µBondapak C_{18} column with a methanol/water (80:20, vol/vol) mobile phase at flow rate of 1 mL/min. This fraction was dried *in vacuo* and redissolved in hexane. The enantiomers of 9(*R,S*)- and 13(*R,S*)-HODE were separated on a Chiralcel OD chiral stationary phase with a hexane/methanol (180:1, vol/vol) mobile phase at a flow rate of 1 mL/minute. The elution order of the enantiomers was determined by coinjection of a racemic mixture of the HODE with 9(*S*)- and 13(*S*)-HODE. Enantiomeric ratios were calculated by comparing the peak areas representing the enantiomers.

RESULTS

The GC–MS characteristics of 9- and 13-HODE ME/trimethylsilyl (TMS) ethers (ME/TMS) can be seen in Figure 2. These compounds are characterized by a peak at *m/z* 382 for the molecular ion ($M^{+\cdot}$), and fragment ions represented by peaks at *m/z* 311 and *m/z* 225 (17). A representative reconstructed total ion chromatogram and mass spectra from analysis of a 100-mg rat mammary gland extract are shown in Figure 3. The O^{18}-labeled internal standards and the analytes have identical retention times. The fragments containing the two ^{18}O-atoms are represented by peaks at *m/z* 386 ($M^{+\cdot}$) and *m/z* 315.

Recovery experiment. Before applying the extraction protocol to the analysis of mammary glands from mice in the diet study, the reliability of the extraction protocol was tested. Recovery experiments were performed using [^{14}C]-13-HODE prepared by soybean lipoxygenase-catalyzed oxygenation of ^{14}C-labeled linoleic acid followed by sodium borohydride reduction of the resulting 13-hydroperoxyoctadecadienoic acid (3,4,18). The labeled HODE was dissolved in ethanol, and its purity was determined by HPLC. The concentration of the standard solution was 3.1 mM with a specific activity of 2.68 µCi/µmole. The labeled HODE was added to pulverized tissue prior to extraction with ethanol. The average recovery was 72% with a 5% relative standard deviation.

Sample handling. To address the possibility that the mammary gland samples might undergo autoxidation during sample workup or during sample storage, a study was designed to evaluate the influence of sample handling on analytical results using a pooled mammary gland tissue sample. The pooled sample was divided into aliquots of 100–200 mg, which were extracted in triplicate. Selected aliquots were ex-

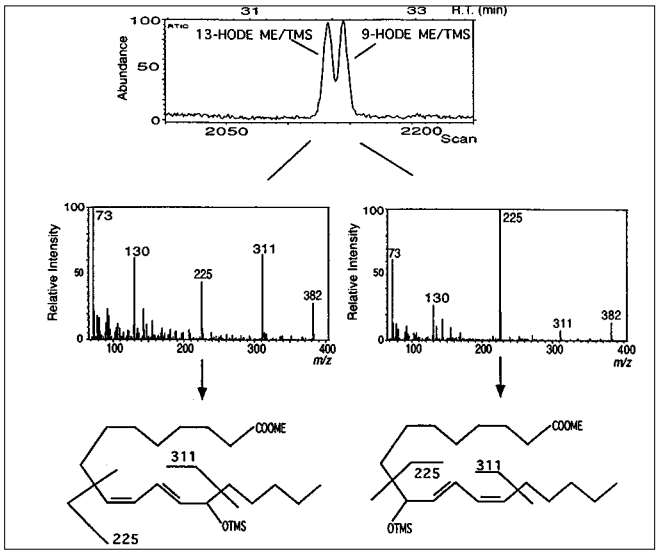

FIG. 2. GC–MS characteristics of 9- and 13-HODE methyl ester/trimethylsilyl (ME/TMS): upper panel is the reconstructed total ion chromatogram; middle panel shows electron ionization mass spectra. See Figure 1 for other abbreviation.

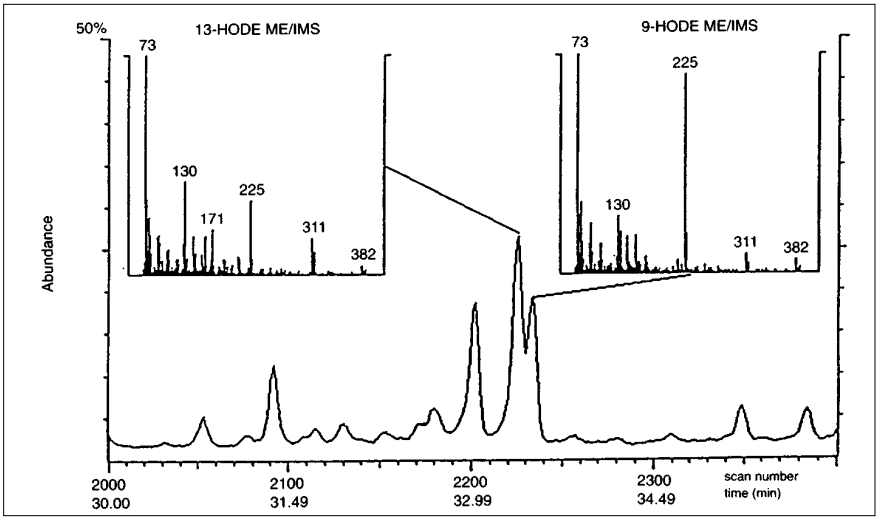

FIG. 3. Reconstructed total ion chromatogram and mass spectra of 9- and 13-HODE ME/TMS from 100 mg mammary gland tissue. See Figures 1 and 2 for abbreviations.

tracted after one-day storage at −80°C, after one-week storage at −20°C, after one-month storage −20°C, and after one-month storage at −80°C. The data from this study (see Table 1) show that extended storage at −20°C does increase the tissue content of both HODE. In contrast, storage at −80°C has relatively little effect on the amount of HODE detected.

We also performed experiments to assess the amount of autoxidation during workup compared to that occurring in the tissue prior to extraction. Samples of mammary gland were extracted in the presence and absence of butylated hydroxytoluene (BHT). The samples were then analyzed by reverse-phase, straight-phase [hexane/0.8% isopropyl alcohol; Lichrosorb Si-60 column (EM Reagents®, Cincinnati, OH)], and chiral-phase chromatography. The results of these experiments are shown in Figure 4. As shown, the presence of BHT during the extraction does reduce the amounts of oxidized linoleate metabolites detected. Nonetheless, in the presence and absence of antioxidant, the *S/R* ratio for 13-HODE indicates a preponderance of the biologically produced isomer relative to the product derived from autoxidation.

Diet study. To evaluate the applicability of the described method for our interest in determining levels of 9- and 13-HODE in mammary gland tissue, a dietary study was performed in which mice were fed one of three isocaloric diets for 5 wk, each diet differing in fat content. The mice were sacrificed, and the amounts of 9- and 13-HODE in the mammary

TABLE 1
Experimentally Determined Concentration of 9- and 13-HODE in Aliquots of a Common Pool of Rodent Mammary Gland Tissue as a Function of Storage Time and Temperatures[a]

Storage conditions	9-HODE	13-HODE
One day (–80°C)	0.43 ± 0.08	1.1 ± 0.1
One month (–80°C)	0.64 ± 0.08	1.0 ± 0.1
One week (–20°C)	0.98 ± 0.06	1.19 ± 0.04
One month (–20°C)	1.3 ± 0.3	1.9 ± 0.4

[a]Values reported as mean ± standard error of the mean, ng HODE/mg tissue. 9-HODE, 9-hydroxyoctadecadienoic acid; 13-HODE, 13-hydroxyoctadecadienoic acid.

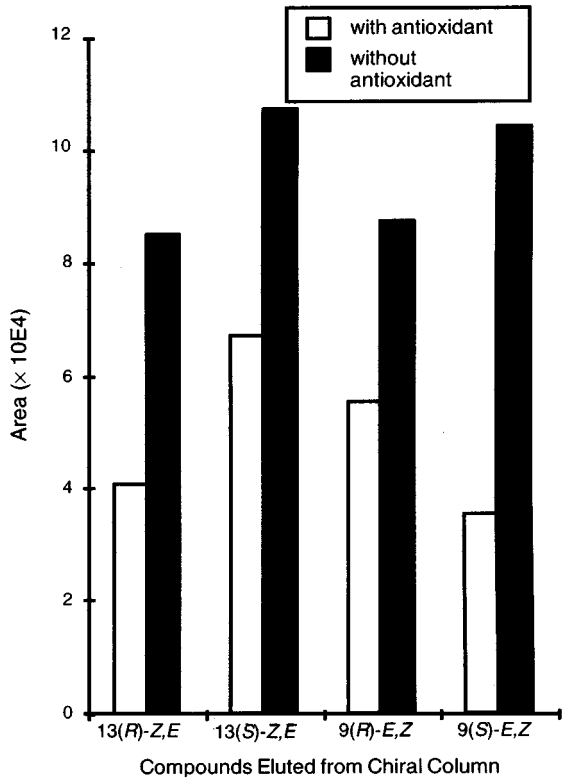

FIG. 4. Enantiomeric abundance of 9- and 13-HODE ME from mammary gland tissue collected from an HPLC separation on a silica column then injected onto a chiral column from samples with and without BHT.

glands were determined. The average (eight mice per diet group) tissue concentrations in the diet study are summarized in Table 2.

An analysis of variance (ANOVA) for the 13-HODE content shows significance ($P < 0.006$) as does an ANOVA for the 9-HODE data ($P < 0.002$). ANOVA examines the main effects of independent variables and potential interactions among them; it does not report which of the pairwise comparisons are statistically different from each other. To determine differences between groups, a Tukey's test for making pairwise comparisons was employed. Tukey's test on both the 9- and 13-HODE data finds no differences between HODE levels in the glands from mice fed the 20% beef tallow diet and from those fed the 5% corn oil diet. However, there are significant differences in the levels of 9- and 13-HODE in the glands of mice fed the 20% corn oil diet vs. the 20% beef tallow diet ($P < 0.05$), and between those fed the 20% corn oil diet vs. the 5% corn oil diet ($P < 0.05$).

Given the high unsaturated fat content of the tissue under investigation, it is necessary to evaluate the relative contributions of autoxidative vs. enzymatic processes to the production of 9- and 13-HODE. Analysis of the distribution of stereoisomers of the products provides a means for identifying product formation by either of the two processes. A representative chromatogram from the enantiomeric separation of the HODE enantiomers from a mammary gland extract is shown in Figure 5. The identity of the 9- and 13-HODE enantiomers was confirmed by coinjection of racemic standards of 9(*R,S*)-(*E,Z*)- and 13(*R,S*)-(*Z,E*)-HODE. In Figure 5, the 13-(*S*)-(*Z,E*) HODE peak also contains 7–10% of the 13(*R*)-(*E,E*) isomer, and the 9(*R*)-(*E,Z*) peak contains a similar percentage of the 13(*S*)-(*E,E*) isomer. These percentages were determined by comparing the distribution of products between mammary gland extracted in the presence and absence of BHT which gives an estimate of autoxidation in the matrix of interest. In spite of the occurrence of low levels of autoxidation, the contribution of the autoxidation products does not alter the conclusions based upon comparison of enantiomeric ratios. In more recent analyses, we have been able to eliminate the contribution of autoxidation products in chiral analysis by separating the *Z,E* and *E,Z* isomers from the *E,E* by straight-phase HPLC. The resulting pure *Z,E* and *E,Z* isomers are then analyzed by chiral-phase chromatography.

Average values for the enantiomeric ratios of 9(*R,S*)-(*E,Z*)- and 13(*R,S*)-(*Z,E*)-HODE are listed in Table 3. The ratios are reported as *S/R* for 13-HODE, but as the reciprocal, *R/S*, for 9-HODE as the *R* enantiomer was more abundant. In calculating the averages for 13-HODE (*S/R*) ratios in the glands of mice fed the 5% corn oil diet and those fed the 20% corn oil diet, individual values were assessed by the Q-test to evaluate their fit within the sample set. The small unlabeled peaks in the chromatogram have not been identified.

TABLE 2
Experimentally Determined Concentrations of 9- and 13-HODE in the Mammary Glands of Female Mice Fed Diets Differing in Fat Content[a]

Diet groups	Number of samples	9-HODE	13-HODE
20% Beef tallow	8	0.2 ± 0.1[a]	0.9 ± 0.2[a]
5% Corn oil	8	0.2 ± 0.1[a]	0.8 ± 0.2[a]
20% Corn oil	8	2.4 ± 0.6[b]	2.7 ± 0.7[b]

[a]a vs. b, $P < 0.05$. Values reported as mean ± standard error of the mean, ng HODE/mg tissue. See Table 1 for abbreviations.

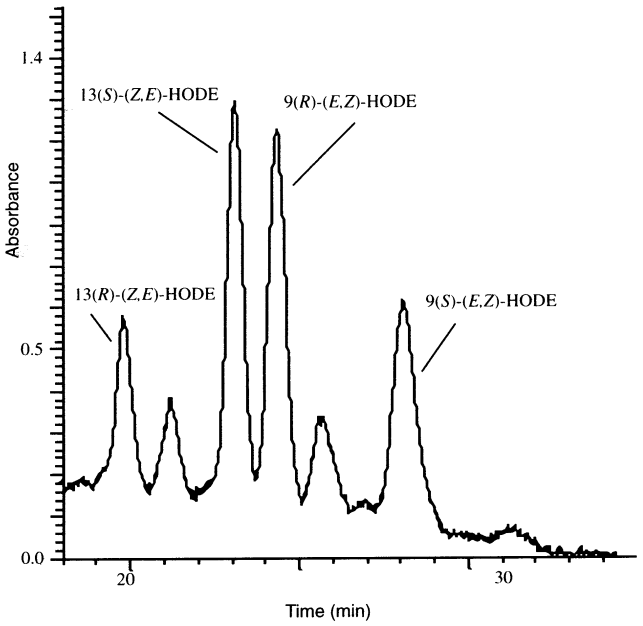

FIG. 5. Chromatogram resulting from enantiomeric separation of 9(*R,S*)-(*E,Z*)- and 13(*R,S*)-(*Z,E*)-HODE methyl esters from a mammary gland extract. Absorbance values in millivolts (mV) at λ = 234 nm. See Figure 1 for abbreviation.

TABLE 3
Distribution of Enantiomers (*R,S*) of 13- and 9-HODE in Mammary Glands of Female Mice Fed Diets Differing in Fat Content[a]

Diet groups	9-HODE (*R/S*)	13-HODE (*S/R*)
20% Beef tallow	1.4 ± 0.2	1.6 ± 0.2
5% Corn oil	1.6 ± 0.4	3 ± 0.5
20% Corn oil	1.4 ± 0.1	3 ± 1

[a]Values reported as mean ± standard error of the mean. See Table 1 for abbreviations.

DISCUSSION

We have developed methodology for extraction and selective detection of 9- and 13-HODE and their enantiomers in rodent mammary gland tissue. The methodology developed in this laboratory showed that application of published extraction protocols for polyunsaturated fatty acids from other tissue systems was not directly applicable to processing mammary gland tissue due to the large quantity of nonhydroxylated fatty acids in the tissue.

The initial application of this methodology to the analysis of mouse mammary gland tissues that had been stored in the freezer at –20°C for one month showed that there were ap-

proximately equal amounts of 9- and 13-HODE in the tissue. The formation of equal amounts of the two isomers suggests formation *via* autoxidation in the tissues either during sample workup or during sample storage. As can be seen from the results in Table 1, the levels of 9- and 13-HODE increase with storage time at −20°C, as does the variance. On the other hand, storage at −80°C for up to one month does not significantly increase the amount of HODE over that determined from freshly extracted tissue. Experiments to evaluate the amount of autoxidation during sample workup indicated some autoxidation in the absence of added antioxidant. However, the workup-derived autoxidation products are not produced in sufficient quantities to alter our conclusion that the majority of HODE production in mammary glands of mice in the diet study was of biological (enzymatic) origin based upon chiral analysis as described below. It is clear that autoxidation products are present in the mammary gland lipids prior to the addition of the antioxidant. However, it is not known whether these autoxidation products arose during removal of the glands from the animal or were present prior to sacrifice.

Application of the methodology to a study of mammary glands from animals fed different levels and types of fat shows that there are significant differences in the levels of 9- and 13-HODE as a function of dietary fat. It was not apparent from these data whether the increased hydroxy fatty acid content arose from biological processes or from autoxidation of lipid pools. To address this issue, we performed analyses of the distribution of stereoisomers of both 9- and 13-HODE. Enzymatic oxidation results in the production of a single stereoisomer whereas autoxidation produces a racemic mixture. The enantiomeric separation of the (*R,S*)-HODE showed that nonracemic mixtures exist in these tissues, with 13(*S*)-(*Z,E*)- and 9(*R*)-(*E,Z*)-HODE being the more abundant isomers. These data suggest formation of 9- and 13-HODE *via* enzymatic rather than autoxidative processes.

Most of the studies reported in the literature have focused on *in vitro* studies of enzymatic oxidation of linoleic acid by various lipoxygenase enzymes (19–21). However, Baer *et al.* (22) reported that the principal *in vivo* oxygenase products of linoleic acid in psoriatic skin scale are 13-HODE (*S/R* = 1.9) and 9-HODE (*R/S* = 2.4). Further, *in vitro* studies by these investigators with cell cultures and radiolabeled linoleic acid produced an enantiopure 13(*S*)-[^{14}C]-hydroxyoctadecadienoic acid. The difference between the *in vivo* and *in vitro* results was explained as follows (23): The discrepancy between the pure (*S*) stereospecificity of the *in vitro*-produced 13-HODE and nonracemic stereospecificity of the *in vivo* compound suggested that there is autoxidation of linoleic acid in the epidermis, resulting in a nonenzymatically derived racemic 13-HODE and an enzymatically produced 13(*S*)-HODE.

It seems likely that a phenomenon similar to that reported by Baer *et al.* (22) is occurring in the mammary gland. In particular, the production of an excess of 13(*S*)-HODE is indicative of enzymatic oxidation. This is especially pronounced in the mice fed the highly unsaturated corn oil diet relative to those fed the more highly saturated beef tallow. In addition, the relatively low constant ratio of 9(*R/S*)-HODE in all dietary groups suggests a significant portion of the 9-HODE is formed by autoxidative processes with only minor contribution from enzymatic oxidation. These data are analogous to those from psoriatic skin in which 13(*S*)- and 9(*R*)-HODE were produced in enantiomeric excess against background racemic products from autoxidation (22). Alternatively, the possibility must be considered that the enzymes responsible for metabolism of either the HODE or the putative hydroperoxide precursors demonstrate stereospecificity. Such a situation could lead to an isomeric excess in the absence of stereospecific formation of a particular isomer; however, since lipoxygenases in general are fairly stereospecific, this seems unlikely.

The method developed in the current work does not include a transesterification or hydrolysis reaction and therefore detects only free fatty acid oxidation products. It is not clear from the present study whether the formation of HODE is from esterified linoleate which is subsequently released or if fatty acid release is followed by oxidation. We are in the process of developing methods for the analysis of esterified HODE which should allow us to address the question of the source of linoleate for HODE production.

In conclusion, an analytical methodology has been developed for analysis of 9- and 13-HODE in rodent mammary gland tissue. GC–MS was used to quantitate the absolute amounts of these metabolites in the tissues. Chiral-phase HPLC analysis was used to provide a qualitative assessment of the degree of enzymatic vs. autoxidative production of HODE. The methodology has been successfully applied to analyses of mouse mammary gland tissues to assess the influence of diets consisting of different amounts and/or types of fat on the production of 9- and 13-HODE. Our experiments demonstrated significant differences between the levels of 9- and 13-HODE in mammary gland tissue from mice in the different diet groups. The enantiomeric distribution of 9(*R,S*)- and 13(*R,S*)-HODE in mouse mammary gland tissue as a function of diet indicates that a combination of enzymatic and nonenzymatic factors contributes to the production of oxidized linoleate products in this tissue. The methodolgy reported in this communication will be critically important in our efforts to understand the importance of oxygenated linoleic acid metabolites in mammary tumorigenic processes.

ACKNOWLEDGMENTS

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Effect of Liposome-Encapsulated Hemoglobin on Triglyceride, Total Cholesterol, Low-Density Lipoprotein, and High-Density Lipoprotein Cholesterol Measurements

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ABSTRACT: The present study investigated the effect of liposome-encapsulated hemoglobin (LEH), an experimental oxygen-carrying resuscitation fluid, on triglyceride, total cholesterol, and low density lipoprotein (LDL), and high density lipoprotein (HDL) cholesterol measurements. *In vivo,* the intravenous infusion of LEH (5.6 mL/kg, $n = 6$) elevated serum triglycerides (+92% vs. baseline, $P < .05$), total cholesterol (+25% vs. baseline, $P < .01$), LDL cholesterol (+72% vs. baseline, $P < .01$) and had no effect on serum HDL cholesterol. In addition, LEH did not alter the elevation in serum triglycerides (+302% vs. baseline, $P < .01$) and LDL cholesterol (+86% vs. baseline, $P < .01$) induced by lipopolysaccharide (3.6 mg/kg, i.v., $n = 6$). *Ex vivo,* measurements of triglycerides and total cholesterol as well as LDL and HDL cholesterol in whole blood from naive rats were not changed by the addition of LEH (0–50%, $n = 6$). *In vitro,* the addition of a fixed concentration of LEH (50%, $n = 6$) to varying concentrations of cholesterol solution (0–50%), or *vice versa,* had no effect on cholesterol determination. It is therefore concluded that LEH only minimally affects serum levels of triglycerides, total cholesterol, LDL cholesterol, and HDL cholesterol and does not interfere with their measurement.

Lipids 32, 377–381 (1997).

Liposome-encapsulated hemoglobin (LEH) is an artificial oxygen-carrying fluid currently under evaluation for the treatment of hemorrhagic shock (for review see Refs. 1,2). The outer layer of LEH, i.e., the phospholipid liposome, consists of 10:9:0.9:0.1 mole percentage of distearoyl phosphatidylcholine/cholesterol/dimyristoyl phosphatidylglycerol/α-tocopherol. Because liposomal cholesterol is known to exchange with cholesterol in lipoproteins (3,4), other liposomes (5–7), and cellular membranes (8,9), it is possible that LEH infusion may affect serum cholesterol and/or lipoprotein levels. Such potential effects could be clinically relevant, especially following the recent demonstration that LEH can modulate endotoxin-induced sepsis (10), in which serum lipoproteins and chylomicrons play a protective role (11–16). To address this issue, the effect of intravenously infused LEH on serum levels of triglycerides and total cholesterol as well as low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol was evaluated in the rat. In addition, *in vitro* and *ex vivo* studies were conducted to determine whether these lipids can be accurately measured in the presence of LEH.

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Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; LEH, liposome-encapsulated hemoglobin; LPS, lipopolysaccharide; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Materials. LEH. LEH produced by the Naval Research Laboratory (Washington, D.C.) was used. The manufacturing method and physicochemical and oxygen-carrying properties of LEH have been previously described (17,18). The phospholipid bilayer of LEH consisted of distearoyl phosphatidylcholine, cholesterol, dimyristoyl phosphatidylglycerol, and α-tocopherol in a mole ratio of 10.0:9.0:0.9:0.1. Lipopolysaccharide (LPS) concentration in LEH was 2.5 ng/mL as measured using the *Limulus* Amebocyte Lysate Pyrochrome Chromogenic Test Kit (Associates of Cape Cod Inc., Woods Hole, MA).

Cholesterol. An aqueous calibrator solution of cholesterol certified at 200 mg/dL by the Center for Disease Control and National Cholesterol Education Program was used (Sigma, St. Louis, MO).

Endotoxin. LPS from *Escherichia coli* 0111:B4 (Sigma) was used as a fresh solution in 0.9% NaCl.

Animals. Male Sprague-Dawley rats (250–300 g: Taconic Farms, Germantown, NY) were kept in individual plastic cages with food and water *ad libitum* at 22°C, 12-h dark/light cycle (0600–1800). Animals were fasted 12 h prior to the experiment.

EXPERIMENTAL PROCEDURES

In vivo. Rats were anesthetized (1% halothane in 100% oxygen) and the femoral artery and vein cannulated for blood sampling and LEH and LPS infusion. Catheters were tun-

neled subcutaneously to the nape of the neck, exteriorized, and secured with a spring wire. Following a 2-h recovery period, a basal blood sample (1.0 mL, exchanged with an equivolume of 0.9% NaCl) for triglycerides as well as total, HDL, and LDL cholesterol assay was collected. LEH ($n = 6$) was infused (1 mL/min) intravenously at 5.6 mL/kg and blood samples for the above assays collected at 15 and 45 min as well as at 1, 1.5, 2.5, and 4.5 h. A similar protocol was repeated with the addition of intravenous bolus infusion of LPS (3.6 mg/kg, $n = 6$) at 30 min after LEH administration.

Ex vivo. Whole blood harvested from naive rats was diluted with increasing amounts of LEH in 8 × 49 mm Ultra-Clear tubes (Beckman, Palo Alto, CA) to give a final concentration of 0–50% in a volume of 1.5 mL. The mixture was centrifuged for 1 h at 100,000 × *g* and serum taken for triglycerides as well as total, LDL, and HDL cholesterol assays. Measured values were compared to values calculated based on the dilution of whole blood *per se*.

In vitro. A volume of 250 µL of the stock cholesterol solution (200 mg/dL) was added to a 250-µL solution containing varying concentrations of LEH (0, 2, 20, 50, and 100% diluted in 0.9% NaCl) ($n = 6$). The mixture was centrifuged in 8 × 49 mm Ultra-Clear tubes for 1 h at 100,000 × *g* and serum taken for total cholesterol assay. Measured values were compared with expected values based on the dilution of the cholesterol solution. A similar experiment was repeated ($n = 6$) with 250 µL of LEH (100%) added to 250-µL solution of varying concentrations of the cholesterol stock solution (0, 2, 20, 50, and 100% diluted in 0.9% NaCl).

ASSAYS

All assays were kindly performed at SmithKline Beecham Clinical Laboratories (Norristown, PA).

Total cholesterol. Total cholesterol was measured using an Olympus cholesterol reagent (Olympus America Inc., Lake Success, NY) which employs a series of coupled enzymatic reactions. In these reactions, free cholesterol hydrolyzed from cholesterol esters is oxidized by cholesterol oxidase to produce cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide reacts to produce a chromophore which is measured biochromatically at 540/600 nm using the Olympus AU5200 Chemistry Analyzer. This procedure is linear to 500 mg/dL and has a sensitivity of 0.6 mg/dL.

LDL cholesterol. LDL cholesterol was assayed utilizing the Direct LDL Immunoseparation Reagent (Genzyme, Cambridge, MA) which removes HDL and VLDL from the sample by using latex beads coated with purified goat polyclonal antisera to specific apolipoproteins. The remaining LDL cholesterol is then quantified using the above enzymatic cholesterol assay with a sensitivity of 7.6 mg/dL.

HDL cholesterol assay. Quantitative separation of HDL was performed using an HDL Precipitating Reagent (DMA, Arlington, TX) followed by an enzymatic cholesterol assay as detailed above. The HDL cholesterol assay is linear to 200 mg/dL.

Triglyceride assay. Triglycerides were measured using the Olympus triglyceride procedure which entails the hydrolysis of triglycerides to free fatty acids and glycerol. Enzymatically phosphorylated glycerol is oxidized to produce hydrogen peroxide which reacts with chromogen 4-aminoantipyrine with the resultant production of a chromophore. The produced chromophore is then measured biochromatically at 520/660 nm using the Olympus AU5200 Chemistry Analyzer and is linear to 1200 mg/dL.

DATA ANALYSIS

Data in text and figures are mean ± SD for the indicated number of animals or samples. One-way analysis of variance followed by Student-Newman-Keuls test was used for statistical analysis. P value of less than 0.05 was considered significant.

RESULTS

In vivo studies. Baseline triglyceride (43 ± 24 mg/dL), total cholesterol (76 ± 17 mg/dL), LDL cholesterol (33 ± 7.3 mg/dL), and HDL cholesterol (47 ± 12 mg/dL) levels did not statistically differ among groups and were in agreement with reported values in the rat (19–22). LEH infusion elevated serum triglyceride (Fig. 1A) as well as total (Fig. 1B) and LDL (Fig. 1C) cholesterol but had no effect on serum HDL cholesterol (Fig. 1D). The intravenous administration of LPS elevated serum triglycerides (146 ± 40 mg/dL, $P < 0.01$ vs. baseline and LPS vehicle, Fig. 1A) and LDL cholesterol (63 ± 17 mg/dL, $P < 0.01$ vs. baseline and LPS vehicle, Fig. 1C) but failed to alter serum total (Fig. 1B) and HDL cholesterol (Fig. 1D). These responses were not affected by pretreatment with LEH (Fig. 1).

Ex vivo studies. The addition of LEH to whole blood from naive rats did not induce statistically significant differences between measured and calculated (based on dilution) values of triglycerides (Fig. 2A), total cholesterol (Fig. 2B), LDL cholesterol (Fig. 2C), and HDL cholesterol (Fig. 2D).

In vitro studies. Cholesterol measurement in solutions containing cholesterol and increasing amounts of LEH (Fig. 3A) did not deviate from expected values calculated based on dilution. Similarly, the addition of increasing concentrations of cholesterol to a fixed amount of LEH did not alter measured cholesterol values as compared to expected values (Fig. 3B).

DISCUSSION

The main findings of the present study are: (i) LEH has a minimal effect on serum levels of triglycerides, HDL cholesterol, LDL cholesterol, and total cholesterol, and (ii) LEH does not interfere with the measurement of these lipids. All experimental paradigms used supported these conclusions. *In vivo*, LEH only marginally elevated serum triglycerides, total, and LDL cholesterol levels and did not affect HDL cholesterol concentration. Also, endotoxin-induced serum levels of triglycerides, HDL cholesterol, LDL cholesterol, and total

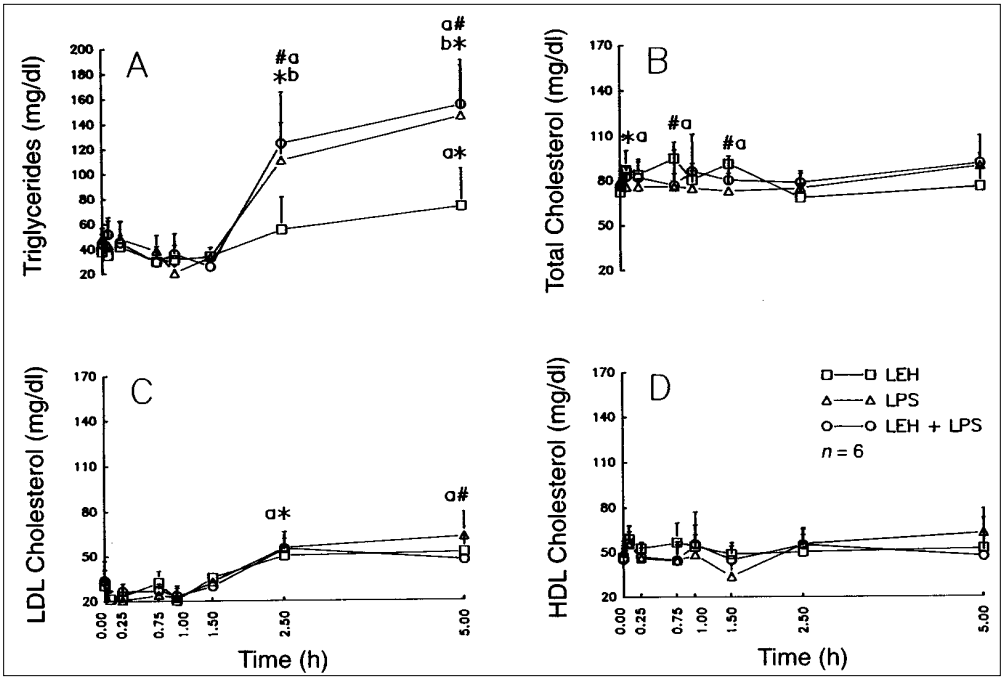

FIG. 1. Effect of liposome-encapsulated hemoglobin (LEH) (10% of calculated blood volume) on basal or lipopolysaccharide (LPS) (3.6 mg/kg, i.v.)-induced serum levels of triglycerides (A), total cholesterol (B), low density lipoprotein (LDL) cholesterol (C), and high density lipoprotein (HDL) cholesterol (D) *in vivo*. $^{*}P < 0.05$; $^{\#}P < 0.01$; a, vs. baseline; b, vs. LEH. Data are presented as mean ± standard deviation.

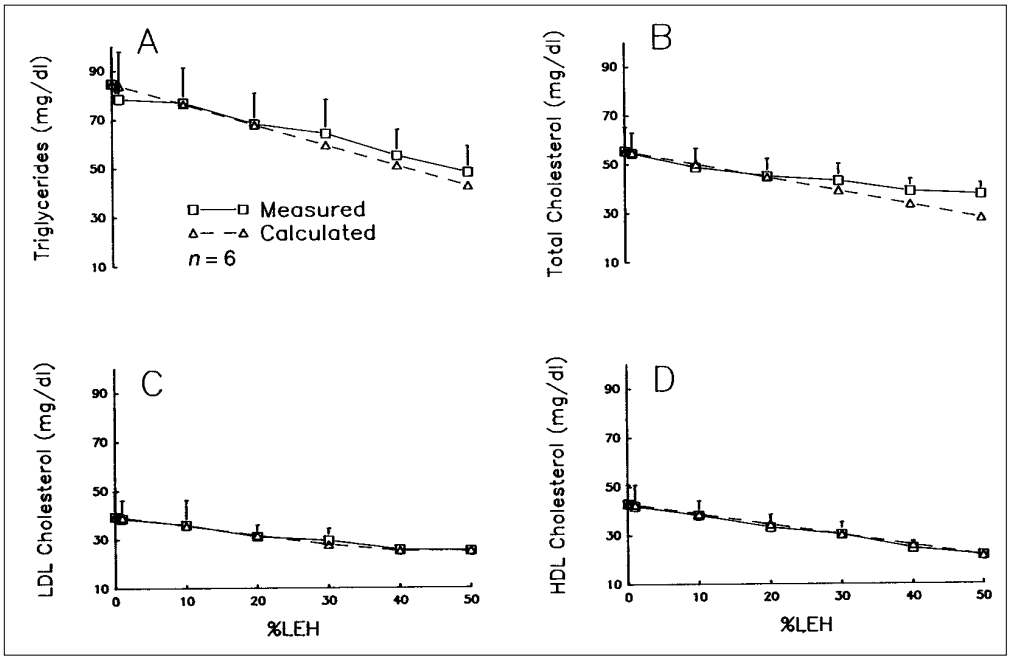

FIG. 2. Effect of LEH (0, 10, 20, 30, 40, and 50%) on triglyceride (A), total cholesterol (B), LDL cholesterol (C), and HDL cholesterol (D) measurements in whole blood harvested from naive rats. Calculated values represent estimates based on the dilution of whole blood *per se*. Data are presented as mean ± standard deviation. See Figure 1 for abbreviations.

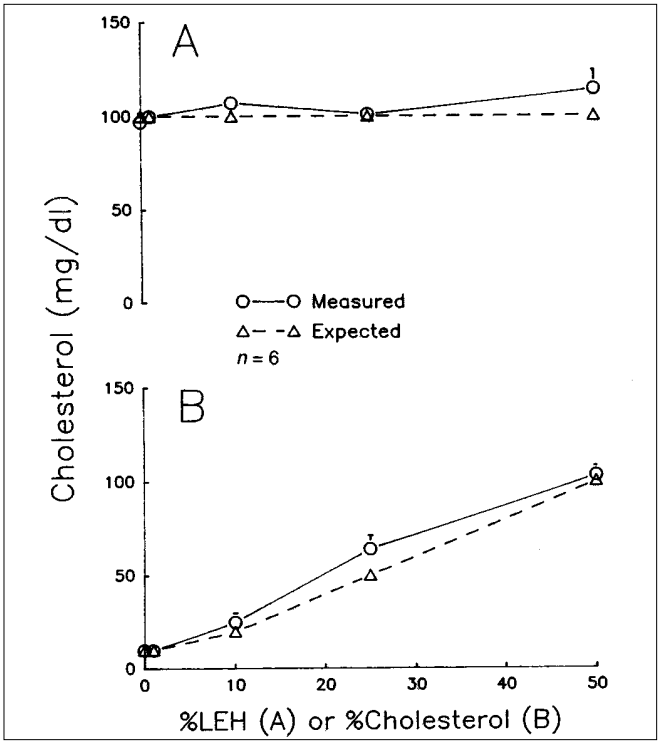

FIG. 3. Effect of LEH on cholesterol measurement *in vitro.* A, LEH (0, 10, 25, and 50%) added to a fixed concentration of aqueous cholesterol (100 mg/dL). B, Cholesterol (0, 10, 25, and 50%) added to a fixed concentration of LEH (50%). Calculated values represent estimates based on dilution *per se.* Data are presented as mean ± standard deviation. See Figure 1 for abbreviation.

cholesterol remained unaffected by LEH infusion. Similarly, when tested in the more restrictive *ex vivo* environment, LEH did not affect the assaying of these compounds in whole blood harvested from naive animals. Furthermore, under the stringent conditions of an isolated *in vitro* system containing only LEH and cholesterol, LEH did not interfere with the determination of cholesterol levels.

The fact that LEH only minimally affected serum levels of triglycerides, HDL cholesterol, and LDL cholesterol and the lack of LEH effect on endotoxin-induced responses of these lipids are clinically relevant. Very recently, LEH has been shown to increase serum TNFα and 24-h mortality in endotoxin-induced sepsis (10) in which VLDL, LDL, HDL, and chylomicrons have been implicated to play a protective role (11–16). Thus, interactions of LEH with these lipids could account for a possible modulatory effect of LEH on the development of sepsis. Such mechanism could be adherence of endotoxin to LEH which competes with endotoxin binding to lipoproteins, the presumed protective mechanism of lipids in sepsis (12–14). However, based on the minor effects of LEH on serum lipoprotein and cholesterol concentrations, it is conceivable that LEH–lipoprotein interactions do not participate in the modulation of sepsis by LEH.

The mechanism(s) of LEH-induced elevation of triglycerides and total and LDL cholesterol are still obscure. Nevertheless, several observations suggest that a likely mechanism could be an exchange of LEH cholesterol with endogenous lipid components. First, cholesterol molecules from liposomes have been previously shown to relocate or exchange with cholesterol in other cellular membranes (8,9). Second, liposome cholesterol is known to freely exchange with cholesterol in chylomicrons as well as very low density lipoprotein, LDL, and HDL cholesterol *ex vivo* (3,4).

In vivo, endotoxin elevated serum triglyceride levels. This is in agreement with numerous studies which characterized the LPS-induced hypertriglyceridemia in a variety of species including the mouse (23,24), rat (25,26), rabbit (27,28), and monkey (29,30). The hypertriglyceridemia observed following endotoxin stimulation was attributed to several mechanisms including increased *de novo* synthesis and mobilization of fatty acids as well as decreased levels of lipoprotein and hepatic triglyceride lipase (31,32). Endotoxin also minimally increased serum concentrations of LDL cholesterol in agreement with earlier hamster (33) and rat (34) studies in which LPS tended to elevate serum levels of both compounds, but no statistical significance was reached. In line with previous reports (20), endotoxin had no effect on serum total and HDL cholesterol within 4.5 h after infusion.

In summary, the present study demonstrated that LEH very mildly elevates serum levels of triglycerides, total cholesterol, and LDL cholesterol and does not interfere with their measurement. Thus, it is conceivable that LEH interaction with these compounds is an unlikely mechanism of LEH modulation of sepsis.

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Bile Acid Metabolism in Young–Old Parabiotic Rats

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ABSTRACT: Serum cholesterol, triglyceride and phospholipid levels, liver cholesterol concentration, bile flow, biliary cholesterol, phospholipid and bile acid secretion rates, fecal sterol and bile acid levels and their bile acid compositions were examined in young–old parabiotic rats and compared with those in young and old control rats and young–young parabiotic rats. Bile acid composition was expressed in terms of the cholic acid group/chenodeoxycholic acid group (CA/CDCA) ratio. Body weight (BW) gain decreased after parabiosis especially in old rats, but the liver weight (g/100 g BW), diet-intake, feces dry weight, liver cholesterol concentration and fecal sterol level were almost the same in all the groups. The biliary bile acid secretion rate was higher and the fecal bile acid level was lower in old rats than those in young rats but both the levels became comparable with those in young rats after parabiosis of old rats with young rats. Young rats, however, showed no changes in these levels after parabiosis. The serum cholesterol level and the biliary and fecal CA/CDCA ratios in old rats were higher than those in young rats but decreased after parabiosis with young rats, although they were still higher than those in young rats. The serum cholesterol level in young rats increased after parabiosis with old rats, but not after parabiosis with young rats, and the fecal bile acid level and the CA/CDCA ratio were not changed in either case. It is concluded from these findings that the serum cholesterol level and the CA/CDCA ratio increased with age and that these increases were prevented after parabiosis with young rats, while young rats, although their serum cholesterol level was increased, showed no increase in the CA/CDCA ratio after parabiosis with old rats.
Lipids 32, 383–390 (1997).

Bile acids are synthesized from cholesterol in the liver, and the major primary bile acids are cholic acid and chenodeoxycholic acid in many animal species. In rats, chenodeoxycholic acid is further transformed to muricholic acids in the liver (1). Therefore, the major bile acids formed in the rat are cholic and β-muricholic acids.

Bile acid synthesis alters with age (2–6). In Wistar rats, cholic acid synthesis increases and chenodeoxycholic acid (mainly β-muricholic acid in the rat) synthesis decreases with aging, while the total bile acid synthesis remains almost constant in rats (3–5), and the cholic acid/chenodeoxycholic acid (CA/CDCA) ratio increases linearly with age (6). Since bile acids are synthesized only in the liver, these changes may reflect age-related alterations in liver function. Therefore, to examine whether the age-related abovementioned changes are directly related to the liver function, we prepared young–old parabiotic rats and examined the changes after parabiosis.

Parabiosis technique has been used in the study of the humoral transmission of many substances including hormones (7) and factors relating to aging (8–13) or immunity (14,15). If the alterations in bile acid metabolism are caused by the age-related changes in humoral factors, the increase in CA synthesis or the decrease in CDCA synthesis in aged rats will be prevented by the parabiosis with young rats, or the bile acid metabolism in young rats will be shifted to an aged state after parabiosis with old rats.

MATERIALS AND METHODS

Male Wistar rats bred in our laboratory were kept in an air-conditioned room (25 ± 1°C, 50–60% humidity) with free access to rat chow (Japan Clea CA-1 diet) and tap water. Parabiosis was performed by the technique described by Shipley (7) under Na·pentobarbital anesthesia (50 mg/kg, i.p.) making young–old and young–young pairs. Control young (4 mon old) and old (14–17 mon old) rats and parabiotic rats were kept individually for 1, 2, and 4 mon after the operation, and the last 2-d feces from the control rats were collected for analysis. In the case of parabiotic rats, a 5% solution of patent blue was given twice orally to one of the rats in each parabiotic pair (1 mL/rat at 1700 on the day before and 1.5 mL/rat on the day) before feces collection, and the 2-d feces not dyed with patent blue were collected. Then, the dye was given to the other rat in each pair 5 d after the first trial and the 2-d feces not dyed were collected for analysis.

At the end of the experiment, the rats were anesthetized with Na·pentobarbital (50 mg/kg, i.p.), and the bile duct was cannulated with PE-10 polyethylene tubing (Clay Adams, Parsippany, NJ) to collect bile for 30 min, while the rectal temperature was controlled at 37°C using an electrically heated plate (16). Blood was then withdrawn from the abdominal aorta and the liver was removed.

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Abbreviations: CA/CDCA, cholic acid/chenodeoxycholic acid group; HDL, high density lipoprotein.

Blood was kept for at least 30 min at room temperature, and serum was obtained by centrifugation at 3000 rpm for 15 min. The serum total cholesterol level was determined with a Determiner-TC 555 Kit (Kyowa Medics Co., Tokyo, Japan), free cholesterol with a Determiner-FC 555 Kit (Kyowa Medics Co.), triglycerides with a Triglysime-V Eiken Kit (Eiken Co., Tokyo, Japan), phospholipids with a phospholipid B-Test Wako (Wako Pure Chemical Industries, Osaka, Japan) and serum high density lipoprotein (HDL)-cholesterol with an HDL-cholesterol test Wako (Wako Pure Chemical Industries). Serum very low density lipoprotein–low density lipoprotein-cholesterol level was taken as the difference between total cholesterol level and HDL-cholesterol level. About one gram of liver tissue was homogenized with chloroform–methanol (2:1, vol/vol, 3×10 mL) and filtered. The liver cholesterol level was determined by a calorimetric method (17).

Bile was extracted by reflux with 40 vol of ethanol at 90°C for 10 min. After filtration, a portion of the extract was evaporated to dryness under a stream of nitrogen. To the residue was added 2 mL of 1.25 N NaOH solution, and it was allowed to undergo hydrolysis at 120°C for 6 h. Sterols were extracted with diethylether, and bile acids were then extracted after acidification with 2 N hydrochloric acid solution (4,18). Feces were lyophilized and ground with a small mill, and fecal sterols and bile acids were determined as reported previously (4,18). Briefly, dried and powdered feces were extracted with absolute ethanol and hydrolyzed in NaOH solution at 120°C under pressure. The sterols and bile acids were then quantified by gas–liquid chromatography. A Hewlett-Packard gas chromatograph Model HP5890A (Palo Alto, CA) equipped with a hydrogen flame-ionization detector, a capillary column (15 m × 0.25 mm, i.d.) coated with DB-17 (J&W Scientific, Folsom, CA), and an HP7673A autoinjector was used in this experiment. Column temperature was kept at 250°C for sterols or programmed to increase from 220–280°C at a rate of 5°C/min for bile acids, the injection port at 280°C, and the detector at 300°C (19).

The amount of diet intake becomes almost constant after maturation, while the body weight and the liver weight continue to increase in rats (4). The fecal bile acid level, which is considered to roughly represent the amount of bile acids synthesized in the liver, remains almost constant after maturation (4), probably due to the dietary cholesterol intake being constant. Based on these facts, the present data on biliary and fecal bile acid levels are expressed in terms of the amounts per rat, instead of those per gram liver weight or body weight.

Statistical analysis was performed by Tukey's test, and a P value less than 0.05 was considered to be significant.

RESULTS

The animals were sacrificed 1, 2, or 4 mon after the parabiosis operation. General profiles of the rats used in this experiment such as rat age at autopsy, final body weight, body weight gain, liver weight, diet intake, and feces dry weight are listed in Table 1. The body weight gain was lower in the

TABLE 1
Age, Final Body Weight, Body Weight Gain, Liver Weight, Diet Intake, and Feces Dry Weight in Control and Parabiotic Rats

	Young			Old		
	1 mon	2 mon	4 mon	1 mon	2 mon	4 mon
Rat age (months) and number of cases						
Control	4 (n = 4)	4 (n = 5)	6 (n = 7)	17 (n = 4)	15 (n = 5)	17 (n = 7)
Young–old parabiosis	4 (n = 4)	4 (n = 5)	6 (n = 6)	17 (n = 4)	14 (n = 5)	17 (n = 6)
Young–young parabiosis		4 (n = 4)				
Final body weight (g)						
Control	391 ± 7.0[a]	381 ± 6.4	442 ± 6.3	406 ± 21.0	418 ± 7.1[c]	381 ± 17.8
Young–old parabiosis	317 ± 11.1[b]	283 ± 9.4[b]	362 ± 5.0[b]	346 ± 12.1[b]	337 ± 5.9[b]	358 ± 16.7
Young–young parabiosis		283 ± 6.2[b]				
Body weight gain (g)						
Control	47 ± 6.4	93 ± 7.8	129 ± 6.4	22 ± 8.5[c]	–20 ± 5.7[c]	–24 ± 25.0
Young–old parabiosis	–13 ± 12.4[b]	–8 ± 10.2[b]	49 ± 8.8[b]	–73 ± 9.2[b,c]	–85 ± 10.2[b,c]	–72 ± 12.7
Young–young parabiosis		12 ± 3.0[b]				
Liver weight (g/100 g body weight)						
Control	3.4 ± 0.05	3.2 ± 0.04	3.5 ± 0.07	4.1 ± 0.26	3.8 ± 0.06[c]	4.0 ± 0.06
Young–old parabiosis	3.4 ± 0.14	4.1 ± 0.18[b]	3.5 ± 0.11	3.6 ± 0.20	3.7 ± 0.18	3.4 ± 0.14[b]
Young–young parabiosis		3.3 ± 0.07				
Diet intake (g/day/rat)						
Control	23 ± 1.5	24 ± 0.7	20 ± 0.5	20 ± 1.7	19 ± 0.8[c]	17 ± 1.1[c]
Young–old parabiosis[d]	42 ± 2.5	32 ± 1.9	34 ± 2.9	42 ± 2.7	40 ± 1.7	32 ± 1.5
Young–young parabiosis[d]		41 ± 1.9				
Feces dry weight (g/day/rat)						
Control	4.9 ± 0.36	5.1 ± 0.15	4.7 ± 0.17	4.2 ± 0.40	5.1 ± 0.21	3.9 ± 0.28[c]
Young–old parabiosis	4.1 ± 0.23	4.0 ± 0.38[b]	3.9 ± 0.17[b]	4.3 ± 0.57	4.7 ± 0.21	3.3 ± 0.31
Young–young parabiosis		4.5 ± 0.32				

[a]Mean ± SEM.
[b]Statistically significant relative to age-matched control rats ($P < 0.05$).
[c]Statistically significant relative to experimental period-matched young rats ($P < 0.05$).
[d]Values by a parabiotic pair of rats.

parabiotic rats, especially in old rats, but the liver weight expressed in terms of g/100 g body weight showed no significant change. Diet intake was also determined, but the value in the case of parabiotic rats was the amount consumed by one pair of rats. Therefore, precise values could not be determined for the parabiotic rats, but individual feces weight could be measured by use of the dye. The feces dry weight seemed to be slightly lowered in parabiotic rats, but the difference was insignificant except in two groups, the young parabiotic rats after 2 and 4 mon, suggesting that the diet intake of parabiotic rats was not markedly decreased.

Serum cholesterol, triglyceride, and phospholipid levels are shown in Table 2. The serum lipid levels in old rats were much higher than those in young rats, but they were markedly decreased after parabiosis, where the triglyceride and phospholipid levels decreased to those of young rats but the cholesterol level was still higher than that of young rats. In contrast, the serum lipid levels in young rats increased after parabiosis with old rats but not after parabiosis with young rats. The serum-esterified cholesterol values in old rats were lower than those in young rats. The value seemed to increase in old rats and decrease in young rats after parabiosis, but the changes were minor.

The changes in serum cholesterol levels are plotted in Figure 1, demonstrating the individual values. The serum cholesterol level in old rats was markedly higher than that in young rats, but the level decreased in old rats and increased in young rats after young–old parabiosis. No increase in the serum cholesterol level was detected in young–young parabiotic rats. Since the serum cholesterol level in young control rats increased 4 mon after the start of the experiment, no difference was found between the control and the parabiotic rats.

Table 3 shows liver cholesterol concentration, bile flow, biliary lipid secretion, and biliary bile acid composition (CA/CDCA ratio). The liver cholesterol concentration was almost constant in all groups showing values of 2.2–2.8 mg/g. The bile flow in old rats was similar to that in young rats but decreased after parabiosis of old rats with young animals, when expressed in terms of volumes per rat. The bile flow in young rats was slightly decreased after parabiosis with old rats, but the decrease was statistically insignificant.

The biliary cholesterol secretion rates were almost the same in all groups. The biliary phospholipid and bile acid secretion rates in old rats were higher than those in young rats, but they decreased after parabiosis with young animals giving similar values to those in young rats.

Rat bile contains various bile acids, but they were classified as belonging to the CA group and CDCA group according to their origin. The CA group was comprised of CA, deoxycholic 7-oxo-deoxycholic acid, 12-oxo-chenodeoxycholic and 12-oxo-lithocholic acids, and the CDCA group was comprised of CDCA, α-muricholic, β-muricholic, lithocholic, hyodeoxycholic, ursodeoxycholic, β-hyocholic (ω-muricholic), 7-oxo-lithocholic, and 6-oxo-lithocholic acids. The ratios of

TABLE 2
Serum Lipid Levels in Control and Parabiotic Rats

	Young			Old		
	1 mon	2 mon	4 mon	1 mon	2 mon	4 mon
Serum total cholesterol (mg/100 mL)						
Control	63 ± 2.2[a]	61 ± 0.8	127 ± 8.2	208 ± 27.8[c]	214 ± 19.5[c]	256 ± 18.5[c]
Young–old parabiosis	76 ± 3.8[b]	134 ± 16.5[b]	127 ± 15.1	78 ± 3.2[b]	148 ± 21.9[b]	140 ± 20.4[b]
Young–young parabiosis		64 ± 2.9				
Serum esterified cholesterol (%)						
Control	74 ± 0.5	83 ± 0.3	77 ± 0.6	71 ± 0.6[c]	72 ± 0.6[c]	71 ± 0.8[c]
Young–old parabiosis	72 ± 1.0	75 ± 0.8[b]	71 ± 2.5	72 ± 0.9	75 ± 0.5[b]	74 ± 0.3[b]
Young–young parabiosis		80 ± 1.4				
VLDL–LDL–cholesterol (mg/100 mL)						
Control	24 ± 2.1	27 ± 0.7	57 ± 5.1	91 ± 15.4	98 ± 10.8[c]	144 ± 14.1[c]
Young–old parabiosis	35 ± 1.3[b]	69 ± 10.3[b]	65 ± 8.1	36 ± 1.9	72 ± 11.7	72 ± 10.4[b]
Young–young parabiosis		34 ± 2.0[b]				
HDL–cholesterol (mg/100 mL)						
Control	39 ± 0.7	34 ± 1.0	70 ± 3.3	118 ± 12.5[c]	116 ± 9.1[c]	112 ± 9.3[c]
Young–old parabiosis	41 ± 3.9	65 ± 6.4[b]	62 ± 7.9	43 ± 4.2[b]	76 ± 10.6[b]	63 ± 8.3[b]
Young–young parabiosis		30 ± 1.3[b]				
Serum triglyceride (mg/100 mL))						
Control	103 ± 13.4	103 ± 2.0	190 ± 20.2	324 ± 81.2	245 ± 27.7[c]	398 ± 27.8[c]
Young–old parabiosis	65 ± 8.9	162 ± 26.5	135 ± 24.6	62 ± 7.6	117 ± 20.8[b]	83 ± 5.3[b]
Young–young parabiosis		62 ± 5.4[b]				
Serum phospholipids (mg/100 mL)						
Control	145 ± 6.6	132 ± 2.4	232 ± 13.2	404 ± 73.7[c]	323 ± 26.7[c]	330 ± 54.3
Young–old parabiosis	141 ± 8.7	236 ± 29.8[b]	217 ± 25.2	145 ± 7.3[b]	237 ± 33.6	212 ± 24.1
Young–young parabiosis		4.5 ± 0.32				

[a]Mean ± SEM.

[b]Statistically significant relative to age-matched control rats ($P < 0.05$).

[c]Statistically significant relative to experimental period-matched young rats ($P < 0.05$). VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

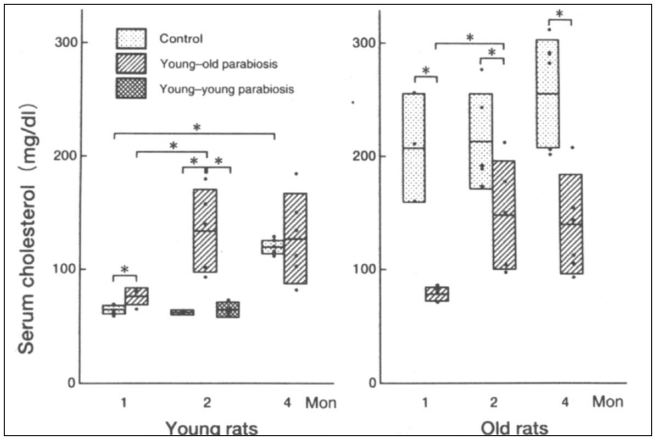

FIG. 1. Serum cholesterol level in control and parabiotic rats.

both bile acid groups (CA/CDCA ratio) were calculated and are listed in Table 3. The CA/CDCA ratio in old rats was higher than that in young rats and decreased after parabiosis with young rats.

Table 4 shows fecal excretion of sterols and bile acids. The major sterols found in rat feces were cholesterol and coprostanol, and their composition ratios were almost the same. Sitosterol was also found, but it was not included in the calculation since this sterol came from the diet and was scarcely absorbed by the rats.

The fecal total sterol excretion was fairly constant in all groups, and the composition ratio of coprostanol, and therefore that of cholesterol, remained almost constant. The fecal bile acid excretion in old rats was lower than that in young rats and seemed to increase slightly after parabiosis, but these differences were mostly insignificant. Interestingly, the composition ratio of the CA group was higher and that of the CDCA group was lower in old rats resulting in an increase in the CA/CDCA ratio. The CA/CDCA ratio in old rats decreased after parabiosis with young rat, but the ratio in young rats was not increased even after parabiosis with old animals.

Changes after parabiosis were presumed to be dependent on the time after the operation, but the differences among the different experimental periods were minor. All the data on biliary and fecal bile acids were therefore gathered without regard to the experimental period, grouped young control rats, young rats in young–old parabiosis, young rats in young–young parabiosis, old control rats and old rats in young–old parabiosis, and are shown in Tables 5 and 6, and Figures 2 and 3.

Table 5 shows the biliary bile acid composition and Table 6 the fecal bile acid composition in the rats grouped as mentioned above. CA and β-muricholic acid were major constituents in the bile, while deoxycholic acid, β-hyocholic acid, and hyodeoxycholic acid were major in the feces. The CA/CDCA ratios in feces were lower than those in bile of the corresponding rat groups.

Figure 2 shows the changes in biliary bile acid secretion

TABLE 3
Liver Cholesterol Level, Bile Flow, Biliary Lipid Levels, and Biliary Bile Acid Composition (CA/CDCA ratio) in Control and Parabiotic Rats

	Young			Old		
	1 mon	2 mon	4 mon	1 mon	2 mon	4 mon
Liver cholesterol (mg/g)						
Control	2.6 ± 0.05[a]	2.7 ± 0.16	2.5 ± 0.05	2.3 ± 0.04[c]	2.5 ± 0.09	2.8 ± 0.09[c]
Young–old parabiosis	2.4 ± 0.09	2.7 ± 0.14	2.6 ± 0.04	2.2 ± 0.05	2.7 ± 0.09	2.6 ± 0.06
Young–young parabiosis		2.6 ± 0.05				
Bile flow (mL/h/rat)						
Control	1.29 ± 0.06	1.25 ± 0.04	1.30 ± 0.03	1.45 ± 0.05	1.37 ± 0.06	1.25 ± 0.06
Young–old parabiosis	1.08 ± 0.09	1.09 ± 0.05	1.25 ± 0.05	1.16 ± 0.04[b]	0.98 ± 0.05[b]	1.06 ± 0.03[b,c]
Young–young parabiosis		1.19 ± 0.11				
Biliary cholesterol (mg/h/rat)						
Control	0.18 ± 0.02	0.16 ± 0.01	0.15 ± 0.01	0.27 ± 0.06	0.19 ± 0.01	0.23 ± 0.01[c]
Young–old parabiosis	0.17 ± 0.04	0.16 ± 0.01	0.18 ± 0.01	0.23 ± 0.02	0.16 ± 0.03	0.20 ± 0.01
Young–young parabiosis		0.16 ± 0.03				
Biliary phospholipids (mg/h/rat)						
Control	4.6 ± 0.41	3.5 ± 0.16	3.5 ± 0.17	6.8 ± 0.49[c]	4.8 ± 0.37[c]	6.8 ± 0.59[c]
Young–old parabiosis	4.3 ± 0.29	3.5 ± 0.29	4.3 ± 0.32	5.1 ± 0.43[b]	4.1 ± 0.91	5.4 ± 0.51
Young–young parabiosis		4.7 ± 0.77				
Biliary bile acids (mg/h/rat)						
Control	17.0 ± 1.28	13.9 ± 0.67	17.0 ± 0.88	25.7 ± 1.72[c]	18.2 ± 1.52[c]	23.9 ± 1.75[c]
Young–old parabiosis	13.8 ± 1.24	11.1 ± 1.05	16.4 ± 2.09	17.9 ± 1.87[b]	10.8 ± 2.35[b]	13.7 ± 1.24[b]
Young–young parabiosis		16.3 ± 2.83				
Biliary CA/CDCA ratio						
Control	0.87 ± 0.04	0.85 ± 0.04	1.08 ± 0.03	1.61 ± 0.16[c]	1.37 ± 0.05[c]	1.69 ± 0.10[c]
Young–old parabiosis	1.00 ± 0.07	0.78 ± 0.05	1.10 ± 0.13	1.26 ± 0.07	1.10 ± 0.06[b,c]	1.27 ± 0.18
Young–young parabiosis		0.87 ± 0.08				

[a]Mean ± SEM.
[b]Statistically significant relative to age-matched control rats ($P < 0.05$).
[c]Statistically significant relative to experimental period-matched young rats ($P < 0.05$); CA/CDCA, cholic acid/chenodeoxycholic acid group.

TABLE 4
Fecal Sterol and Bile Acid Levels and Fecal Bile Acid Composition (CA/CDCA ratio) in Control and Parabiotic Rats

	Young			Old		
	1 mon	2 mon	4 mon	1 mon	2 mon	4 mon
Fecal total sterols (mg/day/rat)						
Control	10.7 ± 1.16[a]	11.2 ± 1.13	10.6 ± 0.71	10.5 ± 1.18	9.4 ± 0.39	11.1 ± 0.52
Young–old parabiosis	11.2 ± 0.67	10.7 ± 2.06	10.7 ± 0.47	10.4 ± 0.63	10.0 ± 0.64	10.5 ± 1.18
Young–young parabiosis		12.1 ± 1.64				
Fecal coprostanol (%)						
Control	55 ± 3.6	54 ± 3.7	53 ± 2.9	62 ± 4.2	38 ± 8.8	46 ± 4.7
Young–old parabiosis	56 ± 3.0	49 ± 11.4	56 ± 2.6	59 ± 5.0	49 ± 5.9	62 ± 4.2
Young–young parabiosis		57 ± 3.2				
Fecal bile acids (mg/day/rat)						
Control	10.6 ± 0.80	10.2 ± 0.79	8.8 ± 0.76	7.0 ± 0.22[c]	7.8 ± 0.85	6.8 ± 0.64
Young–old parabiosis	11.0 ± 1.02	8.4 ± 1.61	8.0 ± 0.55	8.2 ± 0.61[b]	9.5 ± 0.65	7.7 ± 0.91
Young–young parabiosis		10.5 ± 1.56				
Fecal cholic acid group (%)						
Control	35 ± 1.9	31 ± 0.5	38 ± 0.8	47 ± 1.9[c]	45 ± 1.4[c]	43 ± 1.6[c]
Young–old parabiosis	37 ± 0.4	30 ± 1.2	36 ± 1.5	43 ± 2.3	36 ± 1.2[b,c]	36 ± 1.9[b]
Young–young parabiosis		32 ± 1.2				
Fecal chenodeoxycholic acid group (%)						
Control	65 ± 1.9	69 ± 0.5	62 ± 0.8	53 ± 1.9[c]	55 ± 1.4[c]	57 ± 1.6[c]
Young–old parabiosis	63 ± 0.4	70 ± 1.2	64 ± 1.5	57 ± 2.3[c]	65 ± 1.2[b,c]	64 ± 1.9[b]
Young–young parabiosis		69 ± 1.2				
Fecal CA/CDCA ratio						
Control	0.55 ± 0.04	0.46 ± 0.01	0.62 ± 0.02	0.82 ± 0.03[c]	0.82 ± 0.05[c]	0.76 ± 0.05[c]
Young–old parabiosis	0.58 ± 0.01	0.42 ± 0.02	0.57 ± 0.04	0.75 ± 0.07[c]	0.55 ± 0.03[b,c]	0.57 ± 0.05[b]
Young–young parabiosis		0.46 ± 0.03				

[a]Mean ± SEM.
[b]Statistically significant relative to age-matched control rats ($P < 0.05$).
[c]Statistically significant relative to experimental period-matched young rats ($P < 0.05$). See Table 3 for abbreviation.

TABLE 5
Biliary Bile Acid Composition in Control and Parabiotic Rats[a]

	Young rats			Old rats	
	Control	Young–old parabiosis	Young–young parabiosis	Control	Young–old parabiosis
Number of rats	16	15	8	16	15
CA group (%)	46 ± 1.2	43 ± 1.7	46 ± 2.4	58 ± 0.8	52 ± 1.4
Cholic acid	41 ± 1.2	35 ± 0.9	40 ± 2.1	54 ± 0.8	48 ± 1.5
Deoxycholic acid	3 ± 0.2	4 ± 0.2	4 ± 0.3	2 ± 0.2	3 ± 0.2
7-Oxo-deoxycholic acid	1 ± 0.1	5 ± 1.3	3 ± 0.3	1 ± 0.1	1 ± 0.3
12-Oxo-chenodeoxycholic acid	n.d.	n.d.	n.d.	n.d.	n.d.
12-Oxo-lithocholic acid	n.d.	n.d.	n.d.	n.d.	n.d.
CDCA group (%)	54 ± 1.2	57 ± 1.7	54 ± 2.4	42 ± 0.8	48 ± 1.4
Chenodeoxycholic acid	4 ± 0.1	6 ± 0.6	6 ± 0.7	2 ± 0.2	4 ± 0.7
α-Muricholic acid	4 ± 0.2	5 ± 0.5	5 ± 0.7	3 ± 0.2	4 ± 0.4
β-Muricholic acid	37 ± 1.1	36 ± 1.7	35 ± 1.4	32 ± 0.9	30 ± 1.8
β-Hyocholic acid	3 ± 0.2	2 ± 0.1	3 ± 0.7	2 ± 0.2	2 ± 0.3
Ursodeoxycholic acid	1 ± 0.1	1 ± 0.1	2 ± 0.2	<1	1 ± 0.1
Hyodeoxycholic acid	2 ± 0.9	6 ± 0.6	3 ± 1.1	1 ± 0.7	5 ± 1.3
Lithocholic acid	<1	<1	1 ± 0.3	<1	<1
7-Oxo-lithocholic acid	2 ± 0.3	<1	1 ± 0.5	<1	<1
6-Oxo-lithocholic acid	<1	n.d.	<1	<1	<1
CA/CDCA ratio	0.85 ± 0.04	0.78 ± 0.05	0.87 ± 0.08	1.37 ± 0.05	1.10 ± 0.06

[a]See Table 3 for abbreviations; n.d., not detected.

and biliary CA/CDCA ratio. The biliary bile acid secretion in old rats was higher than that in the young, but decreased to the level of young rats after parabiosis with young rats. The biliary CA/CDCA ratio was also high in old rats, and it decreased after parabiosis with young rats, but the level was still higher than that of young rats. The biliary CA/CDCA ratio in young rats, however, was not increased after parabiosis with old rats.

TABLE 6
Fecal Bile Acid Composition in Control and Parabiotic Rats[a]

	Young rats			Old rats	
	Control	Young–old parabiosis	Young–young parabiosis	Control	Young–old parabiosis
Number of rats	16	15	8	16	15
CA group (%)	31 ± 0.5	30 ± 1.2	32 ± 1.2	45 ± 1.4	36 ± 1.2
Cholic acid	<1	<1	<1	2 ± 0.1	<1
Deoxycholic acid	17 ± 1.2	16 ± 0.5	21 ± 2.0	22 ± 1.0	19 ± 1.3
7-Oxo-deoxycholic acid	<1	<1	<1	1 ± 0.3	<1
12-Oxo-chenodeoxycholic acid	4 ± 0.3	3 ± 0.2	3 ± 0.2	4 ± 0.5	3 ± 0.7
12-Oxo-lithocholic acid	11 ± 1.0	9 ± 0.5	8 ± 0.7	16 ± 1.5	13 ± 0.5
CDCA group (%)	69 ± 0.5	70 ± 1.2	69 ± 1.2	55 ± 1.4	65 ± 1.2
Chenodeoxycholic acid	<1	<1	<1	<1	<1
α-Muricholic acid	<1	<1	1 ± 0.2	1 ± 0.3	<1
β-Muricholic acid	5 ± 1.0	7 ± 2.0	11 ± 1.9	16 ± 3.3	9 ± 1.3
β-Hyocholic acid	28 ± 8.7	14 ± 2.8	34 ± 8.4	25 ± 4.1	20 ± 4.7
Ursodeoxycholic acid	n.d.	n.d.	n.d.	n.d.	n.d.
Hyodeoxycholic acid	27 ± 9.3	40 ± 5.2	15 ± 6.2	6 ± 4.0	25 ± 6.0
Lithocholic acid	4 ± 0.3	5 ± 0.6	5 ± 0.8	3 ± 0.3	4 ± 0.9
7-Oxo-lithocholic acid	2 ± 0.3	2 ± 0.3	1 ± 0.3	2 ± 0.5	2 ± 0.4
6-Oxo-lithocholic acid	1 ± 0.3	2 ± 0.7	<1	<1	2 ± 0.7
CA/CDCA ratio	0.46 ± 0.01	0.42 ± 0.02	0.46 ± 0.03	0.82 ± 0.05	0.55 ± 0.03

[a]See Table 3 for abbreviations; n.d., not detected.

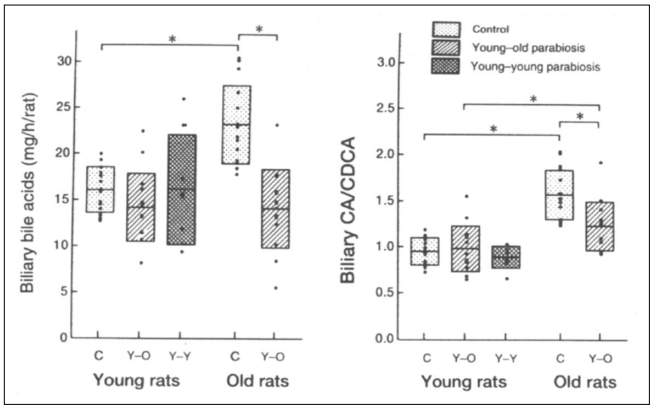

FIG. 2. Biliary bile acids and biliary cholic acid/chenodeoxycholic acid group (CA/CDCA) ratio in control and parabiotic rats. C: Control; Y–O: young–old parabiotic; Y–Y: young–young parabiotic; *statistically significant ($P < 0.05$).

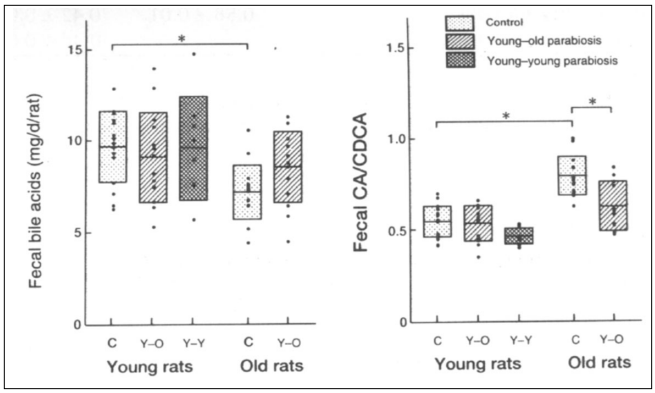

FIG. 3. Fecal bile acids and biliary CA/CDCA ratio in control and parabiotic rats. C: Control; Y–O: young–old parabiotic; Y–Y: young–young parabiotic; *statistically significant ($P < 0.05$). See Figure 2 for abbreviations.

As shown in Figure 3, the level of fecal bile acids in old rats was lower than that in young rats but showed a trend to be increased after parabiosis with young rats. The level of fecal bile acids in young rats was not changed after young–old or young–young parabiosis. The fecal CA/CDCA ratio in old rats was apparently higher than that in young rats, and decreased after parabiosis with young rats, but was still higher than that in the parabiotic young rats.

DISCUSSION

The major primary bile acids in rats are CA and β-muricholic acid, accounting for over 80% of the biliary bile acids in normal rats (4–6,19). In the enterohepatic circulation, these primary bile acids are transformed to secondary bile acids (20). Since these secondary bile acids are absorbed from the intestine to some extent and excreted into bile, biliary bile acids are comprised of various primary and secondary bile acids, although their composition ratios of the secondary bile acids are low. However, the secondary bile acids become major constituents of fecal bile acids (4–6,19). These secondary bile acids can be classified into the CA group and CDCA group according to their origin (21). The compositions of both bile acid groups were explained in the Results section, and changes in the CA/CDCA ratios in bile and feces were discussed in this report.

Bile acids are essential for cholesterol absorption, but *in vivo* the effects of bile acids reflect the actions of CA and not of bile acids related to CDCA (22–24). When cholesterol absorption was examined *in situ* by the small intestinal loop method, taurocholic acid was found to enhance cholesterol

absorption dose-dependently, while tauro-β-muricholic acid showed almost no effect (25). When both of these bile acids were added together to the small intestinal loop, cholesterol absorption was entirely dependent on the concentration of taurocholic acid (25). Therefore, the CA/CDCA ratio was presumed to be an adequate index of the influence of bile acids on cholesterol absorption.

In the present study, the serum cholesterol level and the biliary and fecal CA/CDCA ratios in old rats were higher than those in young rats, and these findings coincided well with our previous results (4–6). CA synthesis increased and CDCA (mainly β-muricholic acid in the rat) synthesis decreased with age, while the total bile acid synthesis remained almost constant in both conventional (3,4,26) and germ-free rats (5,6) when expressed in terms of the amounts per rat. The mechanisms through which CA synthesis increased and CDCA synthesis decreased with age are not yet known, but the increase in serum cholesterol level in old rats was at least partially due to the change in bile acid composition, i.e., an increase in the CA/CDCA ratio, which enhances cholesterol absorption (22–25) for one hand and depresses cholesterol conversion to bile acids or inhibits bile acid synthesis (27–29) for the other hand. Hydrophobic bile acids such as CA and deoxycholic acid show more significant suppression of cholesterol 7α-hydroxylase (27–29) and cholesterol 26-hydroxylase (29) activities than hydrophilic bile acids including muricholic acids and ursodeoxycholic acid.

Since bile acids are synthesized only in the liver, the changes in bile acid composition may be a reflection of changes in liver function. Therefore, biliary and fecal bile acid levels and their compositions, and also serum and liver cholesterol levels, were examined in young–old parabiotic rats in comparison with those in young and old control rats and young–young parabiotic rats in this study.

Young–old parabiotic rats have been used in experiments to elucidate age-related changes in old rats (9–13). Biochemical activities such as Ca^{2+} uptake by the aorta, cholesterol transfer from the serum to the aorta and muscle, or collagenase activity in old rats after parabiosis with young rats seemed to shift closer to those in young control rats, while the activities in the young partner rats remained at the levels of the young controls (9–11). On the other hand, histological characteristics such as hepatic cell size and number in young rats after parabiosis with old rats shifted closer to those in old control rats, and those in old partner rats shifted to those in young controls (12,13).

The biliary and fecal CA/CDCA ratios in old rats decreased after parabiosis with young rats, but they were still higher than those in young controls. The fecal bile acid level which would correspond to the amount of bile acid synthesized in the liver was not changed in parabiotic old rats. Therefore, it is presumed that the liver function responsible for synthesis of bile acids in old rats shifted to that in younger animals after parabiosis with young rats.

On the other hand, the serum cholesterol level and the serum levels of other lipids in old rats decreased after parabiosis, and both very low density lipoprotein–low density lipoprotein–cholesterol and HDL-cholesterol levels decreased almost in parallel with the total cholesterol level, although they fluctuated to an extent after parabiosis. Which humoral factors are involved in the decrease in CA/CDCA ratio in old parabiotic rats could not be defined from the present study, but the serum cholesterol level decreased in these animals.

Young rats, however, showed no changes in either the fecal bile acid level or CA/CDCA ratio after parabiosis with old rats, but their serum cholesterol levels were elevated. Young rats may have a good capacity to tolerate hypercholesterolemia.

It is concluded from these findings that the serum cholesterol level and the CA/CDCA ratio increase with age in rats, and that these increases were prevented after parabiosis with young rats. In contrast, young rats showed no increase in the CA/CDCA ratio after parabiosis with old rats, although the serum cholesterol levels increased.

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Comparative Hypocholesterolemic Effects of Capybara *(Hydrochoerus hydrochaeris dabbenei)* Oil, Horse Oil, and Sardine Oil in Cholesterol-Fed Rats

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ABSTRACT: The hypocholesterolemic efficacy of various polyunsaturated fatty acids was compared in rats given cholesterol-enriched diets. Capybara oil (CO, linoleic + α-linolenic acids), horse oil (HO, α-linolenic acid), and sardine oil (SO, eicosapentaenoic + docosahexaenoic acids) were added to diets at 50 g/kg. The weight gain, food intake, and liver weight in the CO-fed group were significantly higher than those in other groups during the 6-wk experimental period. The serum total and very low density lipoprotein (VLDL) + intermediate density lipoprotein (IDL) + low density lipoprotein (LDL) cholesterol concentrations of the CO-fed and SO-fed groups were significantly lower than in the HO-fed group after 6 wk. The serum high density lipoprotein cholesterol concentration in the SO-fed group was significantly higher than that in the CO-fed and HO-fed groups. The fecal neutral sterol concentration in the CO-fed group was reduced significantly compared with the other groups, and the fecal bile acid concentration in the HO-fed group was significantly higher than that in the SO-fed group. The results of this study demonstrate that CO lowers the serum total cholesterol and VLDL + IDL + LDL-cholesterol concentrations in the presence of excess cholesterol in the diet as well as SO.

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Alteration of the fatty acid profile of dietary fat has been shown to affect several metabolic processes such as those involving plasma lipids and lipoproteins (1,2). In fish oil, the long-chain n-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are active in lowering plasma lipid levels. Fish oils (e.g., salmon oil) affect platelet function by reducing platelet aggregation and adhesion (3), plasma lipids by reducing the plasma concentrations of very low density lipoprotein (VLDL), and triacylglycerols (TG) (4). As for plant oil, there are several reports on the effects of various n-6/n-3 ratios of dietary fatty acids on lipid metabolism (5–9). There also are several reports on the effects of animal oils on lipid metabolism (10,11). Herzberg and Rogerson (10) reported that a tallow diet with fructose reduced serum cholesterol more than a corn oil diet with fructose.

Oil from the rodent capybara, which contains 19.6% linoleic acid (LA, 18:2n-6) and 17.9% α-linolenic acid (ALA, 18:3n-3) is used as a dietary oil in a traditional remedy in Paraguay. The purpose of the present study was to investigate the effect of capybara oil (CO) on the lipid metabolism in rats as compared with sardine oil (SO) and horse oil (HO), which contain abundant EPA and DHA, and abundant ALA, respectively.

MATERIALS AND METHODS

Animals and diets. Male F344/DuCrj rats (4 wk old) were purchased from Charles River Japan Inc. (Yokohama, Japan). Nineteen rats were housed individually in cages in a room controlled for temperature (23 ± 1°C), humidity (60 ± 5%), and lighting (12-h light/dark cycle). The composition of the experimental diet was as follows (wt%): casein, 20; fat, 5; cornstarch, 15; cellulose powder, 5; vitamin mixture, 1 (AIN-76) (12); mineral mixture, 3.5 (AIN-76) (12); choline bitartrate, 0.2; DL-methionine, 0.3; cholesterol, 1; sodium cholate, 0.25; and sucrose to 100. The CO was kindly provided by Dr. S. Kobayashi, Department of Veterinary Medicine, Asunción University (Asunción, Paraguay). SO and HO were obtained from commercial sources. The lipid class of each oil was as follows: CO was composed of 85% TG, 6% sterol, 4% polar lipids, and other components; SO was composed of 84% TG, 6% sterol, 5% polar lipids, and other components; HO was composed of 85% TG, 8% sterol, 2% polar lipids, and other components.

Table 1 shows the fatty acid compositions of the dietary fats. CO, SO, and HO have polyunsaturated fatty acid/satu-

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Abbreviations: AA 20:4n-6, arachidonic acid; ALA 18:3n-3, α-linolenic acid; CO, capybara oil; DHA 22:6n-3, docosahexaenoic acid; EPA 20:5n-3, eicosapentaenoic acid; HDL, high density lipoprotein; HMG–CoA, hydroxymethylglutaryl CoA; HO, horse oil; IDL, intermediate density lipoprotein; LA 18:2n-6, linoleic acid; LDL, low density lipoprotein; PC, phosphatidylcholine; P/S, polyunsaturated fatty acid/saturated fatty acid; SO, sardine oil; TG, triacylglycerol; VLDL, very low density lipoprotein.

TABLE 1
Fatty Acid Compositions of Dietary Fats (wt%)

Fatty acids	Capybara oil	Sardine oil	Horse oil
14:0	3.0	9.6	5.4
16:0	22.7	23.7	26.8
16:1n-7	5.0	9.7	11.7
18:0	4.3	4.5	2.4
18:1n-9	26.5	14.7	31.2
18:2n-6	19.6	2.6	4.3
18:3n-3	17.9	4.0	17.7
20:4n-6	0.7	3.3	0.5
20:5n-3	—	13.5	—
22:6n-3	0.3	14.4	—
n-6/n-3	1.12	0.18	0.27
P/S[a]	1.50	1.14	0.70
Cholesterol (mg/g oil)	0.43	4.02	0.58

[a]Polyunsaturated fatty acid/saturated acid (P/S) = (18:2 + 18:3 + 20:4 + 20:5 + 22:6)/(14:0 + 16:0).

rated fatty acid (P/S) ratios of 1.50, 1.14, and 0.70, respectively, and n-6/n-3 ratios of 1.12, 0.18, and 0.27, respectively. All animals were fed a fat- and cholesterol-enriched diet containing 200 g of palm oil, 10 g of cholesterol, and 2.5 g of sodium cholate/kg for 4 wk as a preliminary diet to create hypercholesterolemic rats. The rats (8 wk old) were divided into a group of seven rats and two groups of six rats each. The rats were allowed free access to experimental diets and water for 6 wk. Body weight and feed consumption were recorded every week. To avoid autooxidation of the fat, each diet was stored at −30°C and freshly prepared each day. All animal procedures described conformed to standard principles (see Ref. 13) .

Analytical procedures. Blood samples (2 mL) were collected weekly between 0800 and 0900 h from the jugular veins of fed rats. The samples were taken into tubes without anticoagulant and, after standing at room temperature for 2 h, sera were prepared by centrifugation at 1500 × *g* for 20 min. At the end of the experimental period of 6 wk, all fecal excretion during 3 d was collected. Fecal dry weights did not differ among groups. The rats were killed by ether inhalation, and the livers quickly removed, washed with cold saline (9 g NaCl/L), blotted dry on filter paper, and weighed before freezing for storage.

Chemical analysis. Total cholesterol and high density lipoprotein (HDL)-cholesterol concentrations in the serum were determined enzymically using commercially available reagent kits (assay kits for the TDX system; Abbott Lab. Co., Irving, TX). The VLDL + intermediate density lipoprotein (IDL) + low density lipoprotein (LDL)-cholesterol concentration was calculated as follows: [VLDL + IDL + LDL-cholesterol] = [total cholesterol] − [HDL-cholesterol].

Total lipids were extracted from liver and feces by a mixture of chloroform/methanol (2:1, vol/vol) (14). The neutral sterol in each total lipid obtained by saponification was acetylated (15) and analyzed by gas–liquid chromatography using a Shimadzu 14A chromatograph (Kyoto, Japan) with a DB17 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) with nitrogen as the carrier gas. Acidic sterols in feces were measured by gas–liquid chromatography following the method of Grundy *et al*. (16). The liver phosphatidylcholine (PC) was separated by two-step single-dimensional thin-layer chromatography (step one, chloroform/methanol 95:5, vol/vol; step two, chloroform/acetone/methanol/acetic acid/water 50:20:15:10:5, by vol). The fatty acids of the PC fraction were methylesterified in HCl/methanol (50 mL/L) for 2 h at 125°C (17) and analyzed by gas–liquid chromatography (Shimadzu 9A) with a 10% DEGS packed column (2 mm × 2.6 m, support shimalite w; Shimadzu) with nitrogen as the carrier gas.

Rat liver enzyme preparation. The liver was homogenized in 2 vol of cold medium containing 50 mM KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.6), and 250 mM sucrose in a Potter-Elvehjem-type homogenizer. After homogenization with only four strokes, the mixture was centrifuged at 1000 × *g* for 10 min, and the supernatant fraction was then centrifuged at 12,000 × *g* for 15 min. The supernatant fraction from this centrifugation was further fractionated by centrifugation at 105,000 × *g* for 60 min, and the resulting pellet was called the microsomal fraction. This microsomal fraction was washed by centrifugation at 12,000 × *g* for 15 min and then at 105,000 × *g* for 60 min in the suspension medium, and finally suspended in 150 mM KCl (pH 7.6) containing 1 mM EDTA.

Determination of cholesterol 7α-hydroxylase (E.C. 1.14.13.17) activity. The procedure followed is described elsewhere (18). One milligram of microsomal protein was added to a 0.1 M potassium phosphate buffer (pH 7.4), 50 mM NaF, 5 mM dithiothreitol, 1 mM EDTA, 200 g/L glycerol and 0.15 g/L 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and the mixture was incubated for 5 min at 37°C. The reaction was initiated by adding NADPH regeneration components, the final concentrations of which were 5 mM sodium isocitrate, 5 mM $MgCl_2$, 0.5 mM NADPH, and 0.075 units of isocitrate dehydrogenase, all in a final reaction volume of 1.0 mL. The reaction mixture was incubated at 37°C for 20 min, unless otherwise indicated, in a shaking (90 strokes/min) water bath. The reaction was terminated by adding 30 μL of 200 g/L sodium cholate, and 1 μg of 7β-hydroxycholesterol as an internal recovery standard. The final reaction was initiated by adding 44 μL of 1 g/L cholesterol oxidase (E.C. 1.1.3.6) in a 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol and 200 g/L glycerol. This reaction mixture was incubated for 10 min at 37°C, and the reaction terminated by adding 2 mL of 950 g/L ethanol. The cholesterol metabolites from this reaction mixture were extracted by adding 6 mL of petroleum ether, vortexing, incubating at 37°C for 3 min, and then centrifuging (1200 × *g*) for 3 min. The upper ethereal layer was collected and dried at 40°C under a nitrogen gas atmosphere. Residues from the various extractions were analyzed by C-18 reverse-phase, high-performance liquid chromatography (Shimadzu 10A), using a Zorbax ODS (4.6 mm × 0.25 m, octadecylsilane, 5–6 μm; MAC-MOD Analytical

Inc., Ford, PA) column equilibrated with acetonitrile/methanol (7:3, vol/vol). The residues were resuspended in 0.1 mL of the same solvent mixture, and 0.02 mL was injected into the column. The metabolites were eluted with the same solvent system at a flow rate of 0.8 mL/min. After 15 min, the flow rate was increased to 2.0 mL/min for 15 min. The amount of product formed was determined by monitoring the absorbance of the effluent at 240 nm and calculating the number of nanomoles from a calibration curve.

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

RESULTS

Food intake, body weight, and liver weight. The results are summarized in Table 2. Body weight gain, food intake, and liver weight were significantly greater in rats fed CO than in the other dietary treatment groups.

Tissue lipid concentration. The total and VLDL + IDL + LDL-cholesterol concentrations in all groups at 6 wk were significantly lower than those at 0 wk. In particular, the total and VLDL + IDL + LDL-cholesterol concentrations in the SO-fed and CO-fed groups were significantly lower than in the HO-fed group at 6 wk (Table 3). There were no significant differences in the HDL-cholesterol concentration of the HO-fed group throughout the experimental period. However, the HDL-cholesterol concentrations in the SO-fed and CO-fed groups were significantly higher than those of the HO-fed group at 6 wk.

Table 4 illustrates the total lipid and cholesterol concentrations in the livers of rats at the end of the 6-wk feeding period. There were no significant differences in the total lipid and liver cholesterol concentrations among the three groups.

TABLE 3
Serum Total, HDL- and VLDL + IDL + LDL-Cholesterol Concentrations in Rats Fed Capybara Oil, Sardine Oil, or Horse Oil for Six Weeks[a]

Diet	0 Wk	3 Wk	6 Wk
Total cholesterol (mmol/L)			
Capybara oil ($n = 7$)	13.7 ± 2.4[a]	5.5 ± 1.1[c,d]	4.8 ± 1.2[d]
Sardine oil ($n = 6$)	13.8 ± 0.8[a]	4.0 ± 0.6[d]	3.9 ± 0.9[d]
Horse oil ($n = 6$)	12.5 ± 0.7[a]	6.8 ± 1.8[c]	8.8 ± 3.1[b]
HDL-cholesterol (mmol/L)			
Capybara oil ($n = 7$)	0.5 ± 0.2[e]	1.2 ± 0.2[c]	2.0 ± 0.6[b]
Sardine oil ($n = 6$)	0.5 ± 0.1[e]	2.3 ± 0.3[a]	1.8 ± 0.6[b]
Horse oil ($n = 6$)	0.7 ± 0.2[d,e]	1.1 ± 0.3[c,d]	1.0 ± 0.2[c,d]
VLDL + IDL + LDL-cholesterol (mmol/L)			
Capybara oil ($n = 7$)	13.3 ± 2.5[a]	4.3 ± 1.0[c,d]	2.8 ± 1.1[d,e]
Sardine oil ($n = 6$)	13.4 ± 0.9[a]	1.6 ± 0.9[e]	2.1 ± 1.3[e]
Horse oil ($n = 6$)	11.9 ± 0.9[a]	5.7 ± 1.8[c]	7.8 ± 3.3[b]

[a]Values are means ± standard deviations. Means within total, HDL, and VLDL + LDL bearing different superscripts are significantly different ($P < 0.05$). VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein.

Cholesterol 7α-hydroxylase activity in the liver. There were no significant differences in the activity of cholesterol 7α-hydroxylase in the microsomal fraction among the three groups (Table 4).

Fatty acid compositions of the tissue. The fatty acid composition of PC in the liver is shown in Table 5. The level of 18:2n-6 was significantly greater, whereas the level of 22:6n-3 was significantly lower in the CO-fed group than in the SO-fed and HO-fed groups. The level of 20:5n-3 in the SO-fed group was significantly higher than in the other groups.

Fecal lipid concentration. Table 6 shows the effects of the dietary oils on fecal neutral sterol and bile acid concentrations in rats at the end of the experimental period. Dietary CO lowered the coprostanol and cholesterol concentrations significantly compared with dietary HO and dietary SO. The excretion of chenodeoxycholic acid was lowered significantly in the CO-fed group.

TABLE 2
Body Weight Gain, Food Intake, and Liver Weight in Rats Fed Capybara Oil, Sardine Oil, or Horse Oil for Six Weeks[a]

	Diet		
Component	Capybara oil ($n = 7$)	Sardine oil ($n = 6$)	Horse oil ($n = 6$)
Body initial weight (g)	195.1 ± 11.6[a]	195.3 ± 7.0[a]	198.1 ± 5.4[a]
Body weight gain (g/6 wk)	115.5 ± 10.2[a]	77.1 ± 10.9[b]	82.5 ± 16.9[b]
Food intake (g/6 wk)	841.9 ± 52.7[a]	749.4 ± 42.5[b]	787.1 ± 47.9[a,b]
Liver weight (wet g/100 g body weight)	6.2 ± 0.2[a]	5.2 ± 0.4[c]	5.7 ± 0.2[b]

[a]Values are means ± standard deviations. Means within the same rows bearing different superscripts are significantly different ($P < 0.05$).

TABLE 4
Liver Total Lipid and Cholesterol Concentrations, and Cholesterol 7α-Hydroxylase Activity in Rats Fed Capybara Oil, Sardine Oil, or Horse Oil for Six Weeks[a]

	Diet		
Component	Capybara oil ($n = 7$)	Sardine oil ($n = 6$)	Horse oil ($n = 6$)
Total lipid (mg/g wet liver)	237 ± 65[a]	187 ± 15[a]	216 ± 33[a]
Cholesterol (μmol/g wet liver)	34 ± 13[a]	40 ± 8[a]	45 ± 14[a]
Cholesterol 7α-hydroxylase (nmol/h per mg protein)	2.94 ± 1.01[a]	3.54 ± 2.03[a]	1.84 ± 1.35[a]

[a]Values are means ± standard deviations. Means within the same rows bearing different superscripts are significantly different ($P < 0.05$).

TABLE 5
Fatty Acid Compositions of Phosphatidylcholine in Liver of Rats Fed Capybara Oil, Sardine Oil, or Horse Oil for Six Weeks[a]

	Diet (wt%)		
Fatty acid	Capybara oil (n = 7)	Sardine oil (n = 6)	Horse oil (n = 6)
16:0	29.4 ± 3.1[a]	29.2 ± 2.0[a]	26.8 ± 1.8[a]
18:0	18.6 ± 3.5[a]	19.7 ± 1.3[a]	19.7 ± 0.7[a]
18:1n-9	23.0 ± 9.9[a]	24.3 ± 1.5[a]	30.5 ± 1.5[a]
18:2n-6	13.4 ± 3.9[a]	4.7 ± 1.4[b]	5.2 ± 0.6[b]
18:3n-3	0.7 ± 0.2[a]	<0.1	0.8 ± 0.1[a]
20:4n-6	9.8 ± 3.6[a]	7.5 ± 0.9[a]	7.4 ± 0.7[a]
20:5n-3	2.6 ± 1.1[b]	8.1 ± 0.4[a]	2.5 ± 0.5[b]
22:6n-3	2.5 ± 1.0[b]	6.5 ± 0.9[a]	7.1 ± 3.5[a]

[a]Values are means ± standard deviations. Means within the same rows bearing different superscripts are significantly different ($P < 0.05$).

DISCUSSION

This study demonstrated that dietary animal oils influence both serum and fecal cholesterol levels and the fatty acid composition of the liver. Fish oils have hypocholesterolemic activity compared with animal and plant oils (19–22). Though the SO contained about 10 times as much cholesterol as the other animal oils, in the diet, which was 1% cholesterol, this difference was negligible. The HO contained abundant 18:3n-3. The high α-linolenate concentration in plant oils such as perilla oil lowers cholesterol levels in the serum and liver more than dietary high-linoleate safflower oil (20). Though the HO lowered the total cholesterol concentration through the experimental period, it had poor hypocholesterolemic function as compared with fish oil and CO. On the other hand, the CO had a hypocholesterolemic function comparable to that of the SO. O'Brien (11) reported that consumption of a beef polar lipid fraction containing 10.6 g LA/100 g polar lipid, as compared with beef tallow containing 3.5 g LA/100 g tallow, seemed to induce hypocholesterolemia. Lee *et al.* (21) reported that serum and liver cholesterol levels were lowered up to a P/S ratio of approximately 2 when the n-6/n-3 ratio was kept relatively constant (5.6–6.4). In this study, the ratios of P/S and n-6/n-3 of the oils that had a hypocholesterolemic function were 1.14 and 0.18 (SO) and 1.50 and 1.12 (CO), respectively. However, the ratios of P/S and n-6/n-3 of the HO were 0.70 and 0.27, respectively. The hypocholesterolemic effect of the CO may be due to the dietary LA/ALA balance being rare in animal oils.

TABLE 6
Fecal Sterol Concentrations in Rats Fed Capybara Oil, Sardine Oil, or Horse Oil for Six Weeks[a]

	Diet (μmol/100 g body weight/d)		
Component	Capybara oil (n = 7)	Sardine oil (n = 6)	Horse oil (n = 6)
Coprostanol	0.29 ± 0.10[b]	0.76 ± 0.40[a]	0.74 ± 0.26[a]
Cholesterol	42.1 ± 14.7[b]	74.4 ± 12.1[a]	66.9 ± 13.1[a]
CA	<0.01	0.50 ± 0.15[b]	1.10 ± 0.34[a]
CDCA	0.18 ± 0.09[b]	0.35 ± 0.10[a]	0.34 ± 0.09[a]
DCA	2.56 ± 1.05[a]	1.28 ± 0.29[b]	2.81 ± 1.23[a]
LCA	0.59 ± 0.30	0.41 ± 0.28	0.50 ± 0.25

[a]Values are means ± standard deviations. Means within the same rows bearing different superscripts are significantly different ($P < 0.05$). The body weights of the three oil groups at 6 wk were not equal: the capybara oil-fed group, 311.6 ± 17.4 g; the sardine oil-fed group, 272.5 ± 15.5 g; the horse oil-fed group, 280.6 ± 13.0 g. CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid.

The lipid compositions and the enzyme activities of the liver and the sterol compositions of feces were examined to elucidate the cholesterol-lowering mechanism of the dietary CO in this study. The body weight gain and liver weight in the CO-fed group were significantly higher than those in the other groups. This may have been due to food intake differences among the three treatment groups. Though there were no significant differences in the cholesterol concentration and total lipid concentration in the liver among the three treatment groups, the total lipid concentration in the CO-fed group increased 9 and 27% as compared with the HO-fed and SO-fed groups, respectively, and the cholesterol concentration in the CO-fed group decreased 15 and 24% compared to the SO-fed and HO-fed groups, respectively. There were no significant differences in the hydroxymethylglutaryl–CoA (HMG–CoA) reductase (E.C. 1.1.1.34) activity (Fukushima, M., unpublished data) and cholesterol 7α-hydroxylase activity in the liver among the three treatment groups. AL-Shurbaji *et al.* (23) have reported that there was no difference between the effects of dietary fish oil and sunflower oil on HMG–CoA reductase and cholesterol 7α-hydroxylase activities in rats, though treatment with dietary n-3 fatty acids resulted in a slight reduction in serum total cholesterol (17%). It has also been reported that administration of fish oil by intravenous infusion results in a marked reduction of serum cholesterol, HMG–CoA reductase activity, and cholesterol 7α-hydroxylase activity compared to intravenous infusion of soybean oil (23). Our study of dietary oils yielded similar results. The reductions of 20:5 and 22:6 levels in the liver PC in the CO-fed group might be due to competition between major pathways of fatty acid biosynthesis by LA or ALA contained in the CO (24).

The HO containing major n-3 fatty acids and poor n-6 fatty acids stimulated cholesterol, coprostanol, and chenodeoxycholic acid secretions (Table 6). However, CO containing major n-6 and n-3 fatty acids suppressed fecal sterol secretions (Table 6) and lowered the serum VLDL + IDL + LDL-cholesterol concentration (Table 3). The results showed that the cholesterol-lowering effect of the CO was not due to fecal excretion of neutral and acidic sterols. It may be that the synthesis of apoliproprotein B-100, which is the major protein component of circulating VLDL + IDL + LDL (25), was lowered in the liver and small intestine, the LDL receptor levels elevated in the liver (26), or that the transfer of cholesterol ester from HDL to VLDL + IDL + LDL was lowered as a result of consumption of CO.

In conclusion, the results of this study demonstrate that CO lowers the serum total cholesterol and VLDL + IDL + LDL-cholesterol concentrations in the presence of excess choles-

terol in the diet. Thus, CO, which is a little-used animal resource containing large amounts of linoleate and α-linolenate, is effective in lowering cholesterol levels in hypercholesterolemic rats.

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High Levels of Dietary Arachidonic Acid Triglyceride Exhibit No Subchronic Toxicity in Rats

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ABSTRACT: Arachidonic acid (AA), an n-6 long-chain polyunsaturated fatty acid (LC-PUFA), serves an important role in the body as a structural fatty acid of many tissues including neurological tissues. It is also a precursor of the n-6 class of eicosanoids and is the most abundant n-6 LC-PUFA found in human breast milk. We have optimized the production of a microfungal source of a triglyceride oil (ARASCO®) which is enriched in AA to about 40% by weight. To establish the safety of this oil as a food, we evaluated the effect of ARASCO® in Sprague-Dawley rats (20/sex/group) gavaged at dose levels of 1.0 and 2.5 g/kg/d for a period of 90 d, paying special attention to any potential neurotoxicity of the oil. Two groups of control animals received either untreated standard laboratory diet (untreated control) or the same diet and vehicle oil at the same dose volume administered to the treated animals (vehicle control). Physical observations, ophthalmoscopic examinations, body weight, food consumption, clinical chemistry, hematology parameters, neurobehavioral assessments, and macroscopic as well as microscopic postmortem evaluations were performed. Tissue fatty acid analyses indicated that the AA levels in the brain, heart, and liver of the high-dose ARASCO®-fed animals increased by 8, 59, and 76%, respectively, indicating that the AA in the oil was readily incorporated into tissue lipids. In spite of this high elevation in tissue AA levels, no developmental, histopathological, or neuropathological differences were seen in the animals administered ARASCO® compared with the vehicle control animals. Being highly enriched in AA, ARASCO® offers the means to study the effect of this fatty acid in experimental settings and in human metabolic studies.
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Arachidonic acid (AA) is a 20-carbon, long-chain polyunsaturated fatty acid (LC-PUFA) with double bonds at the 5, 8, 11, and 14 positions. It is the n-6 precursor of prostaglandins and eicosanoids, and can therefore have a multitude of physiological effects such as mediation of the inflammatory response, regulation of blood pressure, and induction of blood clotting. Low blood levels of AA have been associated with various pathologies such as cirrhosis (1), schizophrenia (2), multiple sclerosis (3), depression (4), and tardive dyskinesia (5). Many of these disorders also exhibit a reduction of docosahexaenoic acid (DHA), suggesting a general Δ6-desaturase deficiency condition. On the other hand, many disorders seem to be associated with some excessive formation of n-6 eicosanoids such as thrombotic stroke (6).

As a structural lipid, AA is especially important in human growth and development. It is provided to the developing fetus *in utero* by the placenta, and to the growing infant postnatally from its mother's breast milk. Most commercially available infant formulas (including all of those available in the United States) do not contain AA, but rather provide a large excess of its metabolic precursor, linoleic acid (LA). In spite of receiving an abundance of dietary LA compared with that found in human milk, formula-fed babies exhibit significantly lower erythrocyte and plasma AA levels compared with breast-fed babies (7). Moreover, Carlson and colleagues (8,9) showed that if preterm babies received a formula supplemented with fish oil containing eicosapentaenoic acid (EPA), the babies' serum AA levels were lowered even further leading to a significant reduction of growth rate. Jensen and colleagues (10,11) later showed that when babies were supplemented with α-linolenic acid (ALA) at levels as high as 4.0% as a precursor to DHA, the consequent elevation of serum EPA was sufficient to depress the babies' AA levels, and growth inhibition was observed. Therefore, the possibility exists that the supplementation of infant formulas with DHA may also need to be accompanied by supplementation with AA at the levels found in human milk. In recognition of the need to match the LC-PUFA composition of human breast milk, the British Nutrition Foundation (BNF), the FAO/WHO Expert Committee on Fats and Oils in Human Nutrition, and the International Society for the Study of Fatty Acids and Lipids (ISSFAL) all have recommended that both AA and DHA be included in the formulas designed to be the sole source of nutrition for infants at levels equivalent to those in human milk (12–14).

ARASCO® (Martek Biosciences Corporation, Columbia, MD) is a triglyceride oil which contains 40% AA, no n-3 fatty acids, and small amounts of other LC-PUFA. It is produced

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; APPT, activated partial thromboplastin time; ARASCO®, arachidonic acid single cell oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FDA, Food and Drug Administration; LA, linoleic acid; LC-PUFA, long-chain polyunsaturated fatty acid.

by conventional fermentation processes from the common soil microfungus *Mortierella alpina* according to Kyle and colleagues (15), and the triglyceride oil product is extracted and processed using unit operations common to the vegetable oil industry. This oil was developed to provide an acceptable source of AA which could be used in infant formula to provide an LC-PUFA composition which more closely matches that of human milk than other sources of AA such as egg yolk. We have previously reported that this oil exhibited no measurable mutagenic or cytotoxic effects, nor could we observe any acute or 28-d subchronic effects at dose levels of 20 g/kg and 2.5 g/kg/d, respectively (16). The purpose of this investigation, as part of a more extensive toxicological study, was to further evaluate the safety of the ARASCO® oil with a standard 90-d toxicity and neurotoxicity assessment in rats. We also studied the incorporation of the AA from ARASCO® into various tissues.

MATERIALS AND METHODS

All toxicological tests were conducted at the laboratories of Huntingdon Life Sciences (formerly Pharmaco LSR) (East Millstone, NJ) in compliance with Part 58 of 21 CFR (FDA Good Laboratory Practice Standards). The "Redbook I" (17) of the Food and Drug Administration (FDA) and the revised "Redbook II" (draft) were used as guidelines for the study design.

Test materials. The material tested was ARASCO® (lot no. A013-DS), a single-cell triglyceride oil produced by *M. alpina* under tightly controlled production procedures (15) following U.S. FDA Good Manufacturing Practices (GMP) for food products. The final product was assayed for elemental composition, chemical characteristics, and fatty acid composition and met previously set specifications (Table 1). Ascorbyl palmitate (0.025%) and mixed natural tocopherol (0.025%) were added to the final product to enhance stability. For this study, additional tocopherol (3 g/kg) was added to ARASCO® to prevent vitamin E deficiency which might occur when high doses of LC-PUFA-containing oils are administered (18). The same amount of extra tocopherol was also added to the high-oleic sunflower oil that was used as the vehicle.

TABLE 1
Fatty Acid Composition (% of total fatty acids) of ARASCO® (test material) and Vehicle Oil (high-oleic sunflower oil)[a]

Fatty acid	ARASCO®	Vehicle oil
12:0	0	0.1
14:0	0.4	0.1
16:0	9.4	4.7
16:1n-7	0.1	0
18:0	10.3	2.2
18:1n-3	8.3	74.5
18:2n-6	6.3	16.7
18:3n-6	3.6	0.4
20:0	0	0.6
20:3n-6	2.8	0
20:4n-6	51.4	0
22:0	1.5	0.2
24:0	0	0.2
22:6	0	0

[a]Used in the 90-d oral gavage toxicity study in rats. ARASCO® (Martek Biosciences Corporation, Columbia, MD).

Animals. Male and female 25-d-old rats (Crl: CD®BR) were obtained from Charles River Breeding Laboratories, Inc. (Stone Ridge, NY) and acclimated for approximately 2 wk before initiation of treatment. At initiation of the test material administration, the rats were approximately 6 wk old, the males weighing from 208 to 273 g, and the females weighing from 146 to 194 g. The animals were examined by a veterinarian during the acclimation period, and those considered unsuitable for the study based on physical examination, body weight, or clinical laboratory data were eliminated prior to assignment to groups. The remaining animals were randomly distributed into four groups of 40 animals each (20 animals/sex) so that the body weight means for each group were comparable. During the treatment period, the animals were housed individually in stainless-steel wire-mesh cages and kept in an environment maintained at 20 to 24°C, 20–74% humidity, and a 12-h light/dark cycle. Certified rodent diet #5002 meal from PMI® Feeds, Inc. (St. Louis, MO) was provided *ad libitum*, except when the animals were fasted. Water was also provided *ad libitum*.

Test material preparation and administration. Test material doses of 1.0 g (low-dose ARASCO®) and 2.5 g (high-dose ARASCO®) ARASCO®/kg/d were prepared by diluting the ARASCO® with high-oleic sunflower oil (lot no. SC 4080; SVO Specialty Products, Eastlake, OH) so as to provide a constant total oil dose of 3 mL/kg/d to each animal based on the most recently recorded body weights. The fat content of the basal diet was 5% (w/w, PMI® #5002). The additional fat bolus gavaged daily varied from 33–52% of the total fat content, depending on the time of the study as the food consumption per body weight (initially approximately 120 g/kg/d) rapidly decreased during the first 4 wk of the study and then leveled out at about 45–55 g/kg/d. Therefore, the additional fat represented 8 to 16% of the energy intake. Fresh dosing solutions were prepared monthly, divided into daily aliquots, and stored frozen. All dose levels were assayed at the time of preparation to verify the concentrations. Daily aliquots were thawed to room temperature before use, and each dose was administered by oral gavage once daily for 91 to 93 d, depending on the day of sacrifice. One group of control animals was untreated, while the other group was dosed with vehicle oil only (high-oleic sunflower oil) (Table 1). The experimental design is outlined in Table 2.

Observation, body weights, and food consumption. The animals (20 animals/sex/group) were observed twice daily, once in the morning and once in the afternoon, for mortality and gross signs of toxicologic effects. A detailed, weekly examination was performed for signs of local or systemic toxicity and tissue masses. The animals underwent an ophthalmoscopic examination prior to test material administration and at termination. Body weights were measured twice prior to

TABLE 2
Experimental Design of the 90-Day Gavage Toxicity Study of ARASCO® in Rats

	Dose (g/kg)	Conc. (g/mL)	Volume (mL/kg)	Initial number of animals		Clinical labs 3 mon		Neurobehavioral studies 0, 5, 9, 13 wk		Neuropathology		Microscopic pathology	
				M	F	M	F	M	F	M	F	M	F
Untreated control[a]	0	0	0	20	20	10	10	10	10	A.R.	A.R.	A.R.	A.R.
Vehicle control	0	0	3[b]	20	20	10	10	10	10	5	5	15	15
Low ARASCO®	1	0.33	3	20	20	10	10	10	10	A.R.	A.R.	A.R.	A.R.
High ARASCO®	2.5	0.83	3	20	20	10	10	10	10	5	5	15	15

[a]Untreated control group received untreated standard laboratory chow. Necropsy was done on all survivors; complete postmortem evaluations were performed on animals which were found dead during the study; A.R. = as required; M = male; F = female. See Table 1 for ARASCO® company supplier.
[b]Control animals received vehicle in the same volume received by the high-dose animals.

test material administration, weekly during treatment, and at termination. Food consumption was measured weekly beginning one week prior to treatment.

Hematology and clinical chemistry. At the end of the treatment period, the animals were fasted overnight, lightly anesthetized with CO_2/O_2, and blood was collected from the retrobulbar orbital plexus from 10 animals per sex per group. Hemoglobin concentration, hematocrit, erythrocyte count, reticulocyte count smear (prepared and stored for possible future examination if deemed necessary), platelet count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, prothrombin time (PT), activated partial thromboplastin time (APTT), total and differential leukocyte counts, and erythrocyte morphology were measured. Serum samples were also analyzed for aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, blood urea nitrogen, fasting glucose, total protein, albumin, globulin, albumin/globulin ratio, creatinine, total bilirubin, sodium, potassium, chloride, calcium, inorganic phosphorous, and γ-glutamyl transferase.

Pathology. At the end of the treatment period, the animals were fasted overnight and sacrificed by exsanguination following carbon dioxide inhalation. A complete postmortem macroscopic examination was performed on all animals. For all survivors not designated for neuropathology (15 animals/sex/group), the following organs were weighed at the scheduled sacrifice: adrenal glands, brain, kidneys, testes with epididymides, thyroid/parathyroid glands (weighed postfixation), liver, ovaries, thymus, and spleen. Paired organs were weighed together. Organ-to-body weight and organ-to-brain weight ratios were calculated. A total of 40 tissues were collected from each animal and preserved in 10% neutral buffered formalin (16). After fixation, the tissues from those animals which died prior to termination of the study and those from animals in the vehicle control and high-dose ARASCO® group were embedded in paraffin, cut at a microtome setting of 4–7 microns, mounted on glass slides, stained with hematoxylin and eosin, and examined by light microscopy. Gross lesions were examined for all animals.

Neuropathology. Five animals per sex per group were anesthetized with an intraperitoneal injection of sodium pentobarbital and transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde/1% glutaraldehyde in the same buffer. The brain, spinal cord (cervical, thoracic, and lumbar segments), sciatic nerve, tibial nerve, and sural nerve were collected from each animal and preserved in 4% paraformaldehyde/1% glutaraldehyde. Slides of each of the above were prepared, and the following regions were examined from the animals in the vehicle control and high-dose ARASCO® group: forebrain, cerebral cortex, hippocampus, basal ganglia, midbrain, cerebellum and pons, medulla, cervical, thoracic and lumbar cord (cross and longitudinal sections), and sciatic, tibial and sural nerves (cross and longitudinal sections).

Neurobehavioral studies. Before initiation of test material administration and at weeks 5, 9, and 13 of treatment, 10 animals per sex per group were monitored for motor activity using an automated Photobeam Activity System (San Diego Instruments, Inc., San Diego, CA). At the same time points, 10 animals per sex per group were also evaluated for neurological condition using a functional observational battery of assessments (Table 3). Most of the assessments were based on qualitatively scaled observations of the animals' behavior/status. Grip strength was measured using a Grip Strength Meter (Columbus Instruments International Corporation, Columbus, OH). Landing foot splay was measured by applying a small dot of paint to each hindpaw, dropping the animal from a height of 2 feet above a flat surface, and measuring the distance between the marks left by the hindpaws. Air-righting ability was assessed by dropping the animal from an upside down position from a height of 2 feet above a container of bedding and observing the landing position.

Tissue fatty acid analysis. In addition to the routine tissue analysis and histology, samples of the brain, heart, and liver were collected from all animals (15/sex/group) not designated for neuropathology evaluations. The organs were frozen in liquid nitrogen and maintained frozen at −80°C until lyophilized. Lipids in the dried organ samples were transmethylated by the following procedure for subsequent gas chromatography (GC) analysis. Each dried sample (50 mg) was weighed into a 15-mL glass tube. To each tube was added 1.5 mL of methanolic base reagent (#3-3080; Supelco, Bellefonte, PA), and the tube was flushed with nitrogen, sealed with a Teflon-lined screw cap, and vortexed. Samples were

TABLE 3
Functional Observational Battery Evaluations of the Neurotoxicological Assessment in the 90-Day Oral Gavage Toxicity Study of ARASCO® in Rats[a]

Home cage evaluations	Handling evaluations
[1]Posture (1-6)	[13]Ease of removal (1-5)
[2]Vocalizations (1-2)	[14]Ease of handling (1-5)
[3]Palpebral closure (1-4)	[15]Chromodacryorrhea (1-2)
	[16]Lacrimation (1-3)
	[17]Coat (1-4)
	[18]Salivation (1-4)
Open field evaluations	Reflex assessments
[4]Gait (1-5)	[19]Approach response (1-6)
[5]Locomotion (1-4)	[20]Tail pinch response (1-6)
[6]Arousal ((1-5)	[21]Finger snap response (1-3)
[7]Piloerection (1-2)	[22]Pupil response (1-2)
[8]Exophthalmia (1-2)	[23]Air righting reflex (1-4)
[9]Fecal boluses (U-#)	
[10]Urine (X-#)	
Grip strength (g)	Landing foot splay (cm)
Abnormal movements	
[11]Convulsions (1-3)	
[12]Tremors (1-3)	

[a]At weeks 0, 5, 9, and 13 (n = 10 for both sexes). Qualitative scales of each evaluation are given in parentheses. [1](sitting or standing normally-crouched), [2,7,8,15](not present-present), [3](eyelids open-eyelids half-closed), [4](normal-forelimbs dragging, unable to support weight), [5](normal-totally impaired), [6](normal-very high, hyperalert), [9](unformed-number of boluses), [10](polyuria-number of pools of urine), [11,12,16](not present-severe), [13,14](very easy-difficult), [17](normal-extremely soiled), [18](not present-extreme), [19](slowly approaches-jumps at object, attacks), [20](normal-freezes), [21](normal, flinches ears-uncommon, jumps), [22](pupil constricts-does not constrict), [23](lands on all four feet-lands on back). See Table 1 for ARASCO® company supplier.

incubated for 15 min in a 65°C water bath, then removed and allowed to cool to room temperature for approximately 10 min. To each tube were added 2 mL of distilled water and 2 mL of *n*-hexane, and the tubes were flushed with nitrogen, capped, and vortexed. Tubes were then centrifuged at 1800 × *g* for 10 min to separate the phases. The top hexane phase containing the methyl esters was dried under nitrogen to a volume of 0.4 mL.

After concentration of the hexane phase, a precipitate was noted in some of the brain samples, resulting in interfering peaks on the chromatograms. This precipitate was removed from these samples by column chromatography or by centrifugation at high speeds. For column chromatography, the hexane phase was loaded onto 300 mg Sep-Pak silica gel columns (Waters/Millipore, Milford, MA) pre-wetted with hexane. The methyl esters were eluted with 2 mL of 10% ether in hexane, dried under nitrogen, and resuspended in 0.4 mL of hexane. Alternatively, the concentrated hexane phase was spun in 1.5 mL microfuge tubes at 13,000 × *g* for 10 min, and the clarified supernatant used for GC analysis. In all samples, the final methyl esters in hexane were transferred to GC autosampler vials, containing sodium sulfate as a drying agent, prior to GC analysis.

Quantitation of the fatty acid methyl esters was performed on a Hewlett-Packard (HP) 5890 Series II GC (Palo Alto, CA). Separations were made on an Omegawax 250 capillary column (30 m, 0.25-mm i.d., 0.025-µm film; Supelco) with helium as the carrier gas at 1.8 mL/min. Each sample (1 µL) was introduced with an HP 7673 autosampler, through a 60:1 split injection at an injector temperature of 260°C. Column temperature was maintained at 205°C throughout the run. Detection was made with a flame-ionization detector at 260°C. Peak identifications were assigned based on retention times of authentic standards. Results for each fatty acid are reported as area percentage of all integrated peaks. Results on >0.10% of total fatty acids are reported. Some brain tissue samples were used for methods development, and thus the number of samples used for final analysis was smaller.

Statistical analysis. Body weight, weight gain, food consumption, hematology and clinical chemistry parameters, organ weights, and organ/body and organ/brain weight ratios were evaluated. Statistical evaluation of equality of means was made by the appropriate one-way analysis of variance technique, followed by a multiple comparison procedure if needed. First, Bartlett's test was performed to determine if groups had equal variance. If the variances were equal, parametric procedures were used; if not, nonparametric procedures were used. The parametric procedures were the standard one-way analysis of variance using the F distribution to assess significance. If significant differences among the means were indicated, Dunnett's test was used to determine which means were significantly different from the control. If a nonparametric procedure for testing of means was needed, the Kruskal-Wallis test was used, and if differences were indicated, a summed rank test (Dunn) was used to determine which treatments differed from control. A statistical test for trend in the dose levels was also performed. In the parametric case (i.e., equal variance), standard regression techniques with a test for trend and lack of fit were used. In the nonparametric case, Jonckheere's test for monotonic trend was used. The test for equal variance (Bartlett's) was conducted at the 1%, two-sided risk level. All other statistical tests were conducted at the 5 and 1%, two-sided risk level. The tissue fatty acid results were evaluated using Student's *t*-test at the 1% two-tailed probability level comparing the high-dose ARASCO® and low-dose ARASCO® groups to the vehicle control group.

For all the tests except tissue fatty acid analysis, the non-vehicle control group was first tested against the vehicle control and, if no difference was found, the vehicle control was used as the true control for the ARASCO® groups.

RESULTS

Mortality. One male from the high-dose ARASCO® group died during the study due to intubation error, and one female from the high-dose ARASCO® group was found dead during the study. In the absence of morphologic changes and clinical signs of toxicity, the deaths of these animals were not attributed to administration of ARASCO®. All other animals survived to the scheduled sacrifice.

Observations, body weights, and food consumption. There were no ARASCO®-related physical observations or ocular

abnormalities noted during the treatment period. Neither the high-dose ARASCO® group nor low-dose ARASCO® group differed from the control group in body weight (Fig. 1) or food consumption.

Hematology and clinical chemistry. There were no ARASCO®-related effects on mean hematology values including PT and APTT at the termination of the study. There were also no differences in the clinical chemistry including liver enzyme activities (Table 4).

Pathology. No ARASCO®-related macroscopic findings were revealed during necropsies of the animals, including signs indicative of micronutrient deficiencies. There were no significant differences in the organ weights between vehicle control and treatment groups. However, the spleen-to-body weight ratios were significantly higher in males in both ARASCO® groups as compared with those in the vehicle control group, but there was no dose response, and the differences were within normal limits compared with historical control data. The differences were not considered to be ARASCO®-related. There were no differences in the spleen-to-brain weight ratios in the same animal groups. No histopathological findings were observed in the tissues examined in either ARASCO® group: adrenal glands, aorta, sternum, bone marrow, brain, epididymides, esophagus, eyes with optic nerve, heart, kidney, intestine, cecum, colon, duodenum, ileum, jejunum, rectum, liver, lungs, lymph nodes, mammary gland, muscle, sciatic nerve, ovary, pancreas, pituitary gland, prostate gland, salivary glands, seminal vesicles, skin, spinal cord, spleen, stomach, testes, thymic region, thyroid/parathyroid glands, trachea, urinary bladder, and uterus.

TABLE 4
Selected Hematology and Clinical Chemistry Results in the 90-Day Oral Gavage Toxicity Study of ARASCO® in Rats at Termination[a]

	Vehicle control[b]	Low ARASCO®[c]	High ARASCO®[d]
Males	*n* = 10	*n* = 10	*n* = 10
PLT (000/(μL)	813 ± 133	711 ± 134	733 ± 189
PT (s)	10.2 ± 0.6	10.5 ± 1.4	11.1 ± 1.4
APTT (s)	29.0 ± 2.7	30.1 ± 6.6	27.7 ± 4.8
WBC (000/(μL)	12.60 ± 3.65	13.44 ± 4.13	14.36 ± 2.73
Neut (000/(μL)	1.45 ± 0.56	1.82 ± 0.80	2.01 ± 0.61
Lymph (000/(μL)	10.45 ± 3.2	10.74 ± 3.32	11.48 ± 2.33
AST (IU/l)	82 ± 0	79 ± 15	92 ± 16
ALT (IU/l)	36 ± 6	39 ± 6	42 ± 9
ALKP (IU/l)	116 ± 24	133 ± 36	122 ± 23
BUN (mg/L)	9.3 ± 1.3	10.0 ± 1.0	11.1 ± 1.4[e]
Females	*n* = 10	*n* = 10	*n* = 10
PLT (000/(μL)	744 ± 111	704 ± 164	761 ± 141
PT (s)	9.4 ± 0.5	9.4 ± 0.3	9.6 ± 0.2
APTT (s)	22.0 ± 2.3	19.6 ± 2.3	22.4 ± 2.7
WBC (000/(μL)	7.97 ± 1.97	7.99 ± 2.36	9.52 ± 1.98
Neutr (000/(μL)	1.23 ± 0.65	0.84 ± 0.53	1.12 ± 0.53
Lymph (000/(μL)	6.33 ± 1.69	6.73 ± 2.23	7.96 ± 1.68
AST (IU/l)	98 ± 1	80 ± 4	88 ± 32
ALT (IU/l)	46 ± 5	43 ± 0	48 ± 41
ALKP (IU/l)	63 ± 5	65 ± 5	61 ± 26
BUN (mg/L)	12.1 ± 2.2	12.3 ± 1.7	13.4 ± 2.2

[a]Means ± SD are given.
[b]3.0 g high-oleic sunflower oil (vehicle)/kg/d gavaged.
[c]1.0 g ARASCO® and 2.0 g vehicle/kg/d gavaged.
[d]2.5 g ARASCO® and 0.5 g vehicle/kg/d gavaged. PLT, platelet count; PT, prothrombin time; APTT, activated partial thromboplastin time; WBC, total leukocyte count; Neut, neutrophil; Lymph, lymphocyte; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALKP, alkaline phosphatase; BUN, blood urea nitrogen.
[e]Significantly different from control ($P < 0.01$). See Table 1 for ARASCO® company supplier.

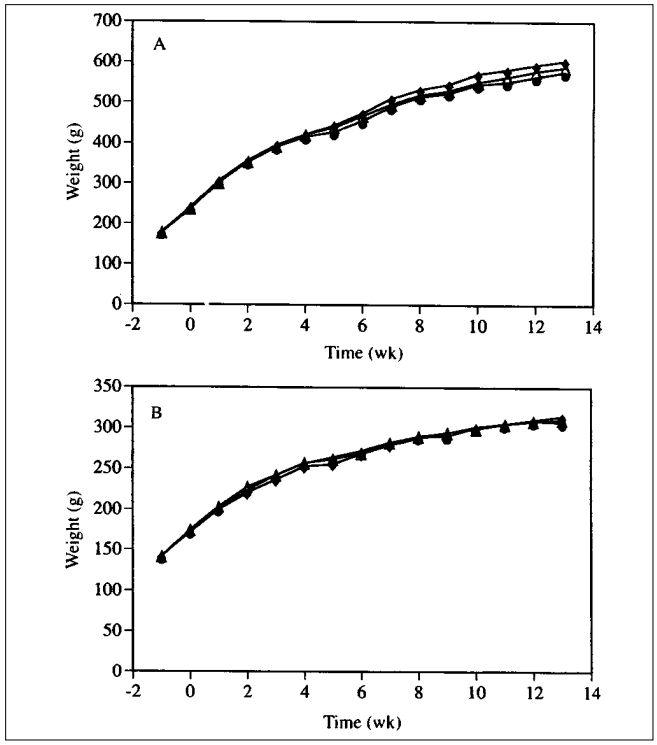

FIG. 1. Weekly body weight gain in rats (A, males; B, females; *n* = 20/sex) gavaged with 3.0 g/kg/d vehicle oil (◆), 1.0 g/kg/d ARASCO® (Martek Biosciences Corporation, Columbia, MD) + 2 g vehicle oil (△) or 2.5 g/kg/d ARASCO® + 0.5 g vehicle oil (●) for 90 d. Group means in grams are shown.

Neuropathology and neurobehavioral studies. There were no ARASCO®-related microscopic findings in the brain, spinal cord, sciatic, tibial, or sural nerves, and no changes in motor activity for either sex in either of the ARASCO® groups. Most of the tests used for the functional observational battery were ranked on a qualitative scale of 1–6 including home cage, open field, handling evaluations, and reflex evaluations. There were no differences between the control and the ARASCO® groups in this neurological testing at the pretest or at weeks 5, 9, and 13. No abnormal movements were observed in any of the groups. Landing foot splay and grip strength for both ARASCO® groups were comparable to the control groups or within the range of normal variation (Table 5).

Tissue fatty acid analysis. The fatty acid compositions of the brain, heart, and liver for high-dose ARASCO®, low-dose ARASCO®, and vehicle control groups are presented in Tables 6–8. Although significant differences in various fatty

TABLE 5
Selected Functional Observational Battery Results in the 90-Day Oral Gavage Toxicity Study of ARASCO® in Rats[a]

	Vehicle control[b]	Low ARASCO®[c]	High ARASCO®[d]
Males	*n* = 10	*n* = 10	*n* = 10
Grip strength/FL (g)	632.0 ± 173.3	786.0 ± 186.9	733.3 ± 141.0
Grip strength/HL (g)	597.0 ± 168.5	701.0 ± 153.6	760.0 ± 182.4
Landing foot splay (cm)	7.6 ± 1.4	8.0 ± 1.5	7.9 ± 1.6
Females	*n* = 10	*n* = 10	*n* = 10
Grip strength/FL (g)	495.0 ± 157.4	559.0 ± 163.7	502.0 ± 143.7
Grip strength/HL (g)	547.0 ± 179.8	455.0 ± 57.8	432.0 ± 90.8
Landing foot splay (cm)	6.6 ± 1.5	6.2 ± 1.8	5.5 ± 1.2

[a]Measured at week 13. Means of first trial ± SD are given
[b]3.0 g high-oleic sunflower oil (vehicle)/kg/d gavaged.
[c]1.0 g ARASCO® and 2.0 g vehicle/kg/d gavaged.
[d]2.5 g ARASCO® and 0.5 g vehicle/kg/d gavaged. FL, forelimb; HL, hindlimb. See Table 1 for ARASCO® company supplier.

acid levels between the groups were seen in all the tissues, the differences were more pronounced in the heart and liver than in the brain.

TABLE 6
Tissue Total Fatty Acids (% of total) at Termination in Brain[a]

Fatty acid	Control[b] (*n* = 20)	Low ARASCO®[c] (*n* = 21)	High ARASCO®[d] (*n* = 19)
13:0	n.d.	0.24 ± 0.26[b]	0.34 ± 0.27[b]
14:0	0.12 ± 0.01	0.12 ± 0.02	0.12 ± 0.01
16:0	19.56 ± 0.46	19.66 ± 0.40	19.54 ± 0.52
16:1	0.40 ± 0.03	0.37 ± 0.15	0.36 ± 0.16
17:0	0.19 ± 0.01	0.20 ±0.01	0.21 ± 0.01
18:0	19.67 ± 0.24	19.84 ± 0.31	19.88 ± 0.26
18:1n-9	21.25 ±0.59	20.54 ± 0.35[b]	20.15 ± 0.79[b]
18:1n-7	4.33 ± 0.14	4.33 ± 0.14	4.40 ± 0.18
18:2	0.77 ± 0.06	0.57 ± 0.04[b]	0.56 ± 0.14[b]
18:3n-3	n.d.	n.d.	n.d.
18:3n-6	n.d.	n.d.	n.d.
20:0	0.37 ±0.05	0.37 ± 0.05	0.38 ± 0.05
20:1	3.06 ± 0.36	2.96 ± 0.26	3.03 ± 0.41
20:2	0.12 ± 0.10	n.d.	0.10 ± 0.06
20:3n-6	0.38 ± 0.05	0.32 ± 0.02[b]	0.34 ± 0.02[a]
20:4	9.93 ± 0.37	10.58 ± 0.37[b]	10.74 ± 0.38[b]
20:5	n.d.	n.d.	n.d.
22:0	0.30 ± 0.03	0.30 ± 0.03	0.31 ± 0.05
22:1	0.33 ± 0.04	0.33 ± 0.04	0.34 ± 0.05
22:4	3.25 ± 0.39	3.60 ± 0.17b	3.46 ± 1.23
22:5n-3	0.23 ± 0.04	0.19 ± 0.03b	0.18 ± 0.01[b]
22:6	12.78 ± 0.86	12.77 ± 0.53	12.17 ± 0.72
24:0	0.29 ± 0.05	0.28 ± 0.05	0.28 ± 0.05

[a]Of rats gavaged with vehicle oil or ARASCO® for 90 d. Means ± SD are given; [a]Significantly different from control ($P < 0.01$); [b]Significantly different from control ($P < 0.001$). n.d. = value is less than 0.10%. See Table 1 for ARASCO® company supplier.
[b]3.0 g vehicle oil/kg/d gavaged.
[c]1.0 g ARASCO® and 2.0 g vehicle/kg/d gavaged.
[d]2.5 g ARASCO® and 0.5 g vehicle/kg/d gavaged.

TABLE 7
Tissue Total Fatty Acids (% of total) at Termination in Liver[a]

Fatty acid	Control[b] (*n* = 30)	Low ARASCO®[c] (*n* = 30)	High ARASCO®[d] (*n* = 30)
13:0	n.d.	n.d.	n.d.
14:0	0.24 ± 0.06	0.22 ± 0.07	0.18 ± 0.05[b]
16:0	17.24 ± 1.11	16.85 ± 1.09	16.18 ± 1.16[a]
16:1	0.51 ±0.21	0.41 ± 0.20	0.31 ± 0.17[b]
17:0	0.31 ± 0.04	0.38 ± 0.05[b]	0.47 ± 0.06[b]
18:0	16.11 ± 3.97	16.55 ± 3.53	19.03 ± 2.97[a]
18:1n-9	16.33 ± 5.14	11.43 ± 3.31[b]	5.66 ± 1.28[b]
18:1n-7	1.53 ± 0.27	1.45 ± 0.20	1.37 ± 0.16[a]
18:2	18.56 ± 2.66	16.21 ± 2.35[b]	14.35 ± 1.87[b]
18:3n-3	0.73 ± 0.21	0.66 ± 0.27	0.60 ± 0.16[a]
18:3n-6	0.32 ± 0.09	0.36 ± 0.13	0.33 ± 0.13
20:0	n.d.	n.d.	n.d.
20:1	0.21 ± 0.13	0.13 ± 0.08	n.d.[b]
20:2	0.21 ± 0.07	0.22 ± 0.04	0.21 ± 0.06
20:3n-6	0.49 ± 0.10	0.46 ± 0.11	0.58 ± 0.14[a]
20:4	17.01 ± 2.56	23.63 ± 2.99[b]	29.94 ± 2.47[b]
20:5	0.78 ± 0.22	0.48 ± 0.17[b]	0.31 ± 0.13[b]
22:0	n.d.	n.d.	n.d.
22:1	n.d.	n.d.	n.d.
22:4	0.45 ± 0.13	1.69 ± 0.69b	1.69 ± 0.63b[b]
22:5n-3	1.25 ± 0.25	1.22 ± 0.23	1.13 ± 0.19
22:6	6.79 ± 2.17	6.51 ± 1.61	6.58 ± 2.01
24:0	n.d.	n.d.	n.d.

[a]See Table 6 for footnotes.

In the brain tissue, ARASCO® supplementation significantly elevated the levels of 13:0 ($P < 0.001$), 22:4 (low-dose only, $P < 0.001$), and AA (high- and low-dose, $P < 0.001$) above the levels found in the vehicle control group. The AA

TABLE 8
Tissue Total Fatty Acids (% of total) at Termination in Heart[a]

Fatty acid	Control[b] (*n* = 20)	Low ARASCO®[c] (*n* = 21)	High ARASCO®[d] (*n* = 19)
13:0	n.d.	n.d.	n.d.
14:0	0.30 ± 0.24	0.28 ± 0.26	0.35 ± 0.34
16:0	10.76 ± 1.13	9.61 ± 1.31[b]	9.37 ± 1.61[b]
16:1	0.36 ± 0.32	0.41 ± 0.36	0.46 ± 0.47
17:0	0.15 ± 0.12	0.19 ± 0.15	0.27 ± 0.18[a]
18:0	15.97 ± 2.58	16.48 ± 2.95	17.04 ± 3.55
18:1n-9	11.19 ± 4.46	7.66 ± 3.71[a]	4.98 ± 2.75[b]
18:1n-7	2.44 ± 0.53	2.34 ± 0.52	2.23 ± 0.49
18:2	22.51 ± 1.76	18.49 ± 2.40[b]	18.27 ± 2.48[b]
18:3n-3	0.20 ± 0.26	0.33 ± 0.33	0.36 ± 0.36
18:3n-6	n.d.	n.d.	n.d.
20:0	n.d.	n.d.	n.d.
20:1	n.d.	n.d.	n.d.
20:2	n.d.	n.d.	n.d.
20:3n-6	0.12 ± 0.19	n.d.	0.20 ± 0.27
20:4	19.69 ± 3.10	27.35 ± 4.38[b]	31.37 ± 5.50[b]
20:5	0.14 ± 0.14	n.d.[b]	n.d.[b]
22:0	n.d.	n.d.	n.d.
22:1	n.d.	n.d.	n.d.
22:4	0.44 ± 0.24	1.32 ± 0.41[b]	1.58 ± 0.52[b]
22:5n-3	2.13 ± 0.53	1.99 ± 0.42	1.58 ± 0.50[b]
22:6	13.31 ± 2.53	12.27 ± 2.60	10.71 ± 2.62[b]
24:0	n.d.	n.d.	n.d.

[a]See Table 6 footnotes.

levels in the brain lipids increased by 8% in the high-dose group and by 7% in the low-dose group. At both levels of supplementation, ARASCO® significantly decreased the levels of 18:1n-9, 18:2n-6, 22:5n-3 (for all $P < 0.001$), and 20:3n-6 ($P < 0.001$ for low-dose, $P < 0.01$ for high-dose).

In the heart tissue, high- and low-dose ARASCO® supplementation resulted in significant decreases in 16:0, 18:1n-9, 18:2n-6, and EPA (for all $P < 0.001$ except for low-dose 18:1n-9, $P < 0.01$). Moreover, a significant increase in AA ($P < 0.001$; 59 and 39%, respectively) and in 22:4 ($P < 0.001$) when compared with the vehicle control group was seen in both dose groups. When used at the high-dose, ARASCO® resulted in significantly decreased levels of 22:5n-3 and DHA ($P < 0.001$ for both) and of 17:0 ($P < 0.01$).

The liver tissue showed the greatest changes as a consequence of the dietary ARASCO®. The 18:1n-9, 18:2n-6, and EPA (for all $P < 0.001$) were significantly decreased in both the high- and low-dose ARASCO® groups while 17:0, 22:4, and AA (for all $P < 0.001$) were significantly increased in both ARASCO® groups (for AA 76 and 39%, respectively). Supplementation with the high dose of ARASCO® resulted in decreased levels of 14:0, 16:0, 16:1, 18:1n-7, 18:3n-3, and 20:1 ($P < 0.001$, $P < 0.01$, $P < 0.001$, $P < 0.01$, $P < 0.01$, $P < 0.001$, respectively) and increased levels of 18:0 and 20:3n-6 ($P < 0.01$, $P < 0.001$, respectively).

DISCUSSION

By using conventional 90-d toxicity testing, we found no signs of toxicity in rats supplemented with ARASCO® at levels as high as 2.5 g/kg/d which corresponds to about 1.0 g AA/kg/d. Dietary AA intake has been estimated to be about 200 mg AA per person per day which is equal to 2.9 mg AA/kg/d for a 70-kg adult (19). Therefore, if directly applicable to humans, this toxicity study represents about a 350-fold safety margin for dietary AA. In addition to the standard battery of toxicity tests, detailed neurobehavioral studies further confirmed the absence of neurotoxicity. Analysis of the tissues from animals in this study also showed that AA from ARASCO® is readily bioavailable. Considerable concern has been expressed about the adverse physiological response caused by AA based on a previous study which showed augmented prostaglandin synthesis in humans after high-dose ethyl arachidonate administration (20). We have demonstrated in this study that high doses of ARASCO® in rats were nontoxic, and also that high doses of AA (up to 1.0 g/kg/d) had no adverse physiological effects when given orally to rats for subchronic periods. The rat is a generally recognized toxicological model with a 70% prediction rate of human toxicity (21).

AA is an important structural lipid, and its role in nutrition has been associated with growth, and *via* modulation of eicosanoid production, immunological status and platelet function. One of the most profound effects that has been reported is that lowered blood levels of AA inhibit growth in premature infants (8). This study showed that an overabundance of AA does not have any effect on food intake or growth in rats. Furthermore, even though *in vitro* studies have shown AA-induced apoptosis in neutrophils (22), this *in vivo* study showed no changes in the differential count of white blood cells. Also, no changes were found in the activity of liver enzymes, reflecting no dysfunction of the liver as a result of the high-AA doses.

Nelson and colleagues (23–27) have studied the effects of dietary supplementation of 4.5 g ARASCO®/d in humans and found no change in platelet aggregation. Platelet aggregation was not measured in this study, but we found no changes in PT and APTT reflecting normal liver function in producing clotting factors. PT and APTT are generally believed to be more sensitive measurements in this respect than platelet count (28).

ARASCO® has been shown to be readily bioavailable in humans (29) as well as in various animal models (16,30,31). In humans it is difficult to analyze the tissue levels of a specific fatty acid, and AA level in blood phospholipids is usually used as a surrogate marker of tissue AA. Animal studies, on the other hand, provide the possibility to also analyze various tissue levels of AA in response to dietary intervention. In a previous study (16), we had used the same doses of ARASCO® (1.0 g AA/kg/d) as in this study (high-dose group) and found similar changes in tissue fatty acids within 28 d. The longer supplementation period in this study (90 d) resulted in only a few additional significant changes in brain fatty acid levels (18:1n-9, 20:3n-6, 20:4n-6, 22:5n-3, 20:4n-6) which were not observed after the 28-d supplementation. This suggests that the brain is somewhat resistant to changes in membrane fatty acids. In contrast, clear dose responses in the liver and heart tissue could be seen (see Tables 7 and 8). The significant decrease in the ARASCO® groups in the levels of 18:1n-9 may be a result of feedback inhibition, whereas the increase in 22:4 is likely to be the result of elongation of AA. Whelan and colleagues (32) have proposed that AA is preferentially incorporated into the tissue when both the precursor 18:2n-6 and AA are available. Our study supports this idea, showing a significant decrease in 18:2n-6 in all the tissues while AA is significantly increased. The decrease in the EPA concentrations in the liver and heart tissues in the ARASCO®-supplemented groups reflects the inverse relationship of dietary AA and EPA on tissue levels as also shown in previous studies (33,34).

Greater changes in the fatty acid composition of liver and heart tissue compared with brain were measured, suggesting that these changes reflect more of an acute effect resembling the effect seen in plasma lipids. Nelson and coworkers (26) have shown that various plasma lipid fractions exhibit marked differences in the degree of arachidonic acid enrichment after ARASCO® supplementation, whereas no differences could be measured in the adipose tissue fatty acid composition. This may be due to tissue selectivity for AA incorporation or simply due to the dilution of the dietary AA in a large pool of adipose tissue. In studies with patients having peroxisomal disorder where ARASCO® has been given orally alone or in combination with a DHA-rich algal oil, the level of AA was

normalized in plasma phospholipids and in red blood cells within 6 wk (Raymond, G., personal communication).

In conclusion, we have demonstrated that very high levels of dietary AA (1.0 g/kg/d), when provided orally to rats as the triglyceride ARASCO® over a period of 90 d, resulted in no significant toxicological, physiological, or behavioral consequences. This dose level represents about a 350-fold safety margin compared with normal dietary AA in humans with no observable effects.

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COMMUNICATION

Possible Fatty Acyl Pheromone Precursors in *Spodoptera littoralis*. Search for 11- and 12-Hydroxytetradecanoic Acids in the Pheromone Gland

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ABSTRACT: Lipidic extracts of *Spodoptera littoralis* pheromone glands submitted to acid methanolysis using: (i) sulfuric acid/methanol/benzene (0.1:4:2, by vol) at 90°C for 1 h; (ii) 12 N HCl/methanol (1:2, vol/vol) at 90°C for 1 h, or (iii) 14% BF_3–MeOH at 90°C for 1 h did not reveal the presence of either 11- or 12-hydroxytetradecanoic acid in the extracts, as concluded from the gas chromatography–mass spectrometry analyses. Under the above methanolysis conditions, a synthetic sample of methyl (14,14,14-$^2H_3$) 12-hydroxytetradecanoate remained unaltered. These results may indicate that formation of (*E*)-11-tetradecenoic acid from tetradecanoic acid does not occur in the pheromone gland by dehydration of an intermediate hydroxyacid. Acid methanolysis of a lipidic extract using BF_3–MeOH led to the formation of a mixture of methoxy fatty acid methyl esters, identified by gas chromatography–mass spectrometry. These methoxy derivatives should arise from BF_3-catalyzed addition of methanol to the double bond of the natural monounsaturated fatty acyl derivatives present in the gland. Thus, under the same conditions, a synthetic sample of methyl (*Z*)-11-tetradecenoate was partially transformed into methyl 11-methoxytetradecanoate and methyl 12-methoxytetradecanoate. This reaction might be a useful alternative procedure to obtain methoxy derivatives of olefins, which are very helpful for the structural characterization of the parent alkenes.

Lipids 32, 407–412 (1997).

The analysis of pheromone gland composition in different moth species has revealed the occurrence of unusual fatty acids that have been proposed as precursors of pheromone components (1). The identification of these intermediates is normally carried out by gas chromatography coupled to mass spectrometry (GC–MS) analysis of the fatty acid methyl esters (FAME) obtained by either acidic or basic methanolysis of pheromone gland lipids.

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Abbreviations: FAME, fatty acid methyl esters; GC–MS, gas–chromatography coupled–mass spectrometry; 11-MeO-16:Me, methyl 11-methoxyhexadecanoate; 12-MeO-16:Me, methyl 12-methoxyhexadecanoate; 9-MeO-14:Me, methyl 9-methoxytetradecanoate; 10-MeO-14:Me, methyl 10-methoxytetradecanoate; 11-MeO-14:Me, methyl 11-methoxytetradecanoate; 12-MeO-14:Me, methyl 12-methoxytetradecanoate; *Z*11-14:Me, methyl (*Z*)-11-tetradecenoate.

The characteristic fatty acids involved in the biosynthetic pathway of *Spodoptera littoralis* sex pheromone blend were identified as tetradecanoic, (*Z*)-9-tetradecenoic, (*Z*) and (*E*)-11-tetradecenoic, (*Z*,*E*)-9,11-tetradecadienoic, hexadecanoic, and (*Z*)-11-hexadecenoic acids (2). The biochemical routes through which these compounds are interconverted to give rise to the pheromone blend were also elucidated (3). In this biosynthetic pathway, the main pheromone component, (*Z*,*E*)-9,11-tetradecadienyl acetate, is formed from tetradecanoic acid by sequential *E*-11 and *Z*-9 desaturations followed by reduction and acetylation.

Whereas *Z* desaturases have been the focus of attention in studies of biosynthesis of fatty acids and their derivatives, the *E* desaturation reaction has been so far less explored. In this context, we undertook a study aimed at investigating the mechanism of formation of (*E*)-11-tetradecenoic acid from tetradecanoic acid in *S. littoralis*. A possible mechanism would involve isomerization of the parent (*Z*)-fatty acid (4). However, recent work carried out in our laboratories has demonstrated that isomerization of (*Z*)-11-tetradecenoic acid to the (*E*)-compound does not occur in *S. littoralis* pheromone glands (Navarro, I., Mas, E., Fabrias, G., and Camps, F., submitted for publication). In light of the rising evidence (5,6) that desaturases and hydroxylases are structurally related at the protein level, *de novo* synthesis of (*E*)-fatty acids might involve initial biohydroxylation of a saturated precursor followed by dehydration of the resulting hydroxy fatty acyl derivative. Thus, according to this approach, formation of (*E*)-11-tetradecenoic acid from tetradecanoic acid might occur through dehydration of either 11- or 12-hydroxytetradecanoic acids. In this context, the aim of this article was the analysis of *S. littoralis* pheromone gland extracts for the presence of the above-mentioned hydroxy fatty acids, since their detection would support the hydroxylation–dehydration proposed mechanism.

EXPERIMENTAL PROCEDURES

Acid methanolysis of standard *Z*11-14:Me (100 ng) was performed by reaction in sealed tubes with 100 μL each of a solution of: (i), sulfuric acid/methanol/benzene (0.1:4:2, by vol) at 90°C for 1 h; (ii), 6 N HCl/methanol (1:2, vol/vol) at 90°C

for 1 h; or (iii) 14% BF_3–MeOH (Merck, Darmstadt, Germany) at 90°C for 1 h. After the reaction, solvents were evaporated and the residues were extracted with 0.5 mL of hexane, and the organic extracts were stored at −30°C until analysis.

To obtain the lipidic extracts, groups of three pheromone glands were soaked in 100 µL of chloroform/methanol (2:1, vol/vol) overnight at −4°C. Samples to be methanolyzed were evaporated to apparent dryness with a stream of nitrogen, and acidic methanolysis was performed by reaction of the resulting residues under conditions (i), (ii) and (iii) as indicated above and, additionally, by treatment with 100 µL of 14% BF_3–MeOH at 4°C for 15 h.

Analyses were performed by electron–ionization GC–MS, at 70 eV, using a Fisons (Rodano, Italy) gas chromatograph (8000 series) coupled to a Fisons MD-800 mass selective detector. The system was equipped with an apolar Hewlett-Packard (Palo Alto, CA) HP-1 capillary column (30 m × 0.20 mm i.d.), which was programmed from 80 to 300°C at 12°C/min after a 2-min delay (analyses of methoxy fatty acids) or a polar SGE (Ringwood Vic, Australia) BP-20 capillary column (30 m × 0.20 mm i.d.), which was programmed from 80 to 260°C at 5°C/min after a 2-min delay (analyses of hydroxy FAME). Injections were carried out in the splitless mode, with a purge of 40 s after injection. Helium was used as carrier gas at a pressure of 12 psi.

RESULTS AND DISCUSSION

Preliminary analyses of base-methanolyzed lipidic extracts of *S. littoralis* pheromone glands did not reveal the presence of

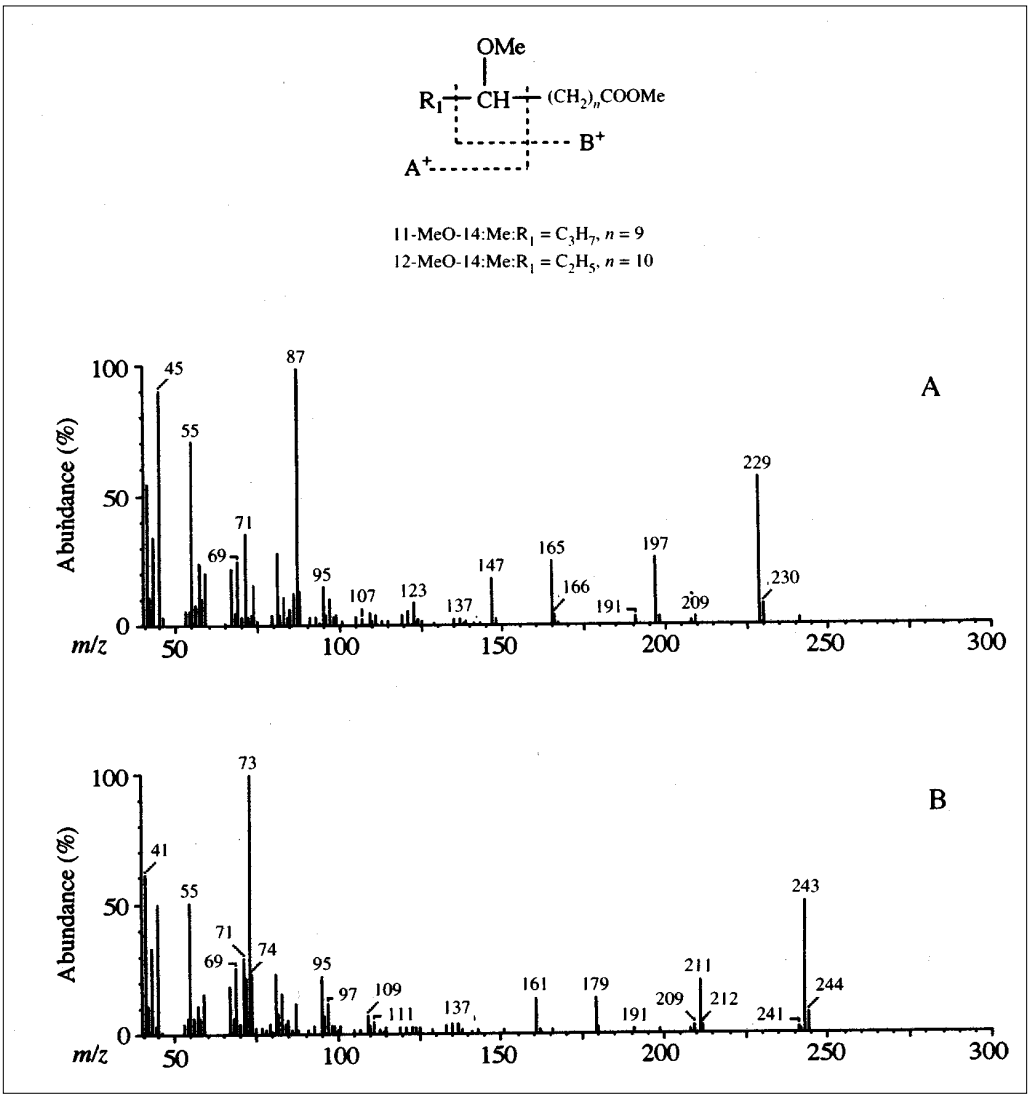

FIG. 1. Mass spectra of methyl 11-methoxytetradecanoate (A) and methyl 12-methoxytetradecanoate (B), formed by treatment of methyl (*Z*)-11-tetradecenoate with a solution of 14% BF_3–MeOH (90°C/1 h) as indicated in the Experimental Procedures section. Structure of ions A^+ and B^+ is also shown. Diagnostic fragments are: 11-MeO-14:Me, 87 (ion A^+) and 229 (ion B^+) and 12-MeO-14:Me, 73 (ion A^+) and 243 (ion B^+). 11-MeO-14:Me, methyl 11-methoxytetradecanoate and 12-MeO-14:Me, methyl 12-methoxytetradecanoate.

either 11- or 12-hydroxytetradecanoic acid in the extracts (data not shown). However, although the acyl moiety of the putative hydroxy fatty acyl derivative is probably ester-linked and is thus hydrolyzable under basic conditions, the hydroxyl group might not be free, but bound to a biological derivative only cleavable under acidic conditions, such as an ether lipid, for example. Therefore, we planned to submit the lipidic extract to acidic methanolysis, which would release both the carboxyl moiety and the hydroxyl group. Acid-catalyzed addition of water to double bonds is a common reaction of olefins (7). Since *S. littoralis* pheromone glands contain both (*E*) and (*Z*)-11-tetradecenoyl moieties (2,3), such reaction might give methyl 11-hydroxy and 12-hydroxytetradecanoates as artifacts. Therefore, to avoid misleading results, we first examined the outcome of acid methanolysis of synthetic methyl (*Z*)-11-tetradecenoate (*Z*11-14:Me) under the following conditions: (i) sulfuric acid/methanol/benzene (0.1:4:2, by vol) at 90°C for 1 h (8); (ii) 6 N HCl/MeOH (1:2, vol/vol) at 90°C for 1 h; and (iii) 14% BF_3–MeOH at 90°C for 1 h. Starting material remained unaltered under conditions (i) and (ii), as concluded from the absence in the GC–MS traces of any additional peak besides that corresponding to *Z*11-14:Me. However, treatment of *Z*11-14:Me (100 ng) with 14% BF_3–MeOH led to the formation of small amounts of methyl 11-methoxytetradecanoate (11-MeO-14:Me, 7 ng) and 12-methoxytetradecanoate (12-MeO-14:Me, 6 ng), which were identified by MS (Fig. 1). Methoxy alkanes and derivatives thereof show mass spectra with diagnostically significant fragment ions A^+ and B^+ (see Fig. 1) arising from carbon–carbon cleavage on either side of the methoxy group, with the charge being retained on the oxygen-containing fragment (9,10). Since, as indicated above, almost the same amounts of each methoxy derivative, 11-MeO-14:Me and 12-MeO-14:Me, are formed from *Z*11-14:Me, these artifacts arise probably from a nonregioselective BF_3-catalyzed addition of methanol to the *Z*11-14:Me double bond. Formation of methoxy derivatives as artifacts has also been reported in the acidic methanolysis of cyclopropane fatty acids (11).

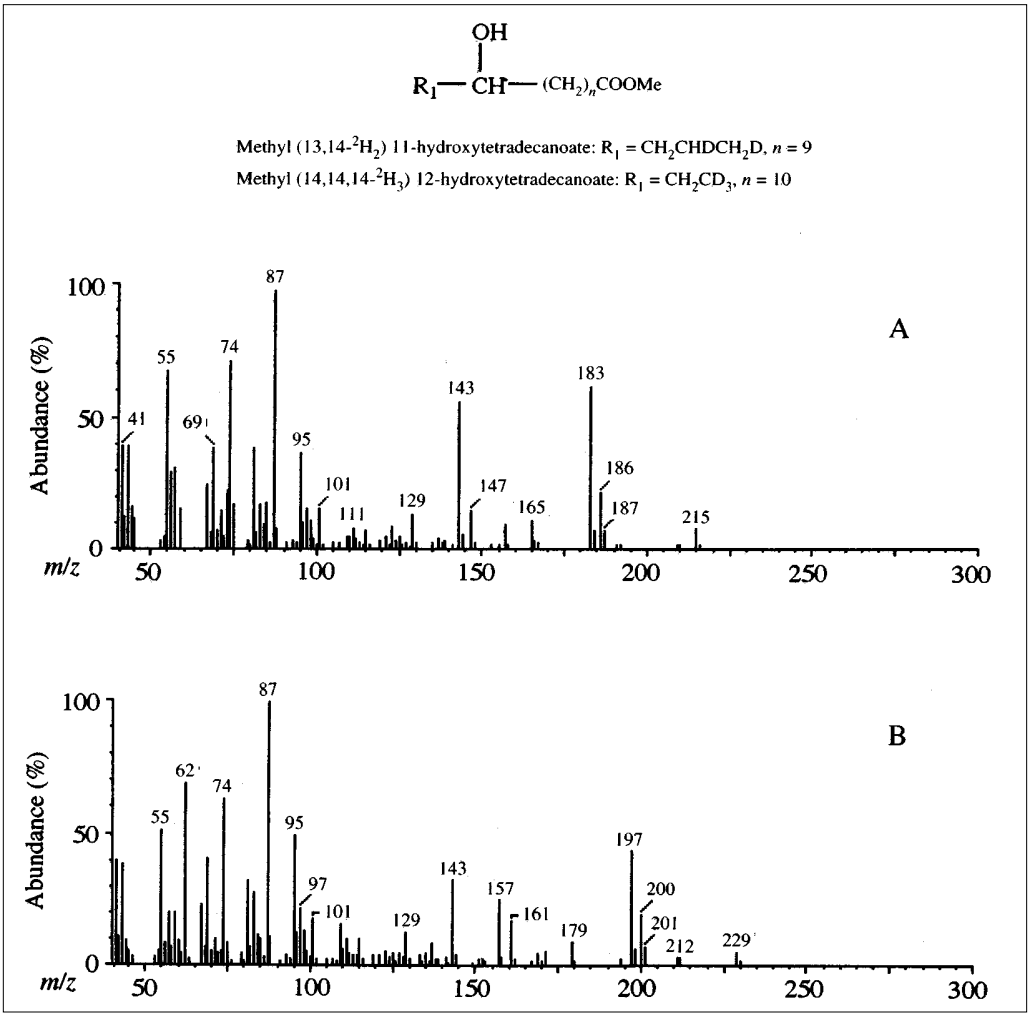

FIG. 2. Mass spectra of methyl (13,14-$^2H_2$) 11-hydroxytetradecanoate (A) and methyl (14,14,14-$^2H_3$) 12-hydroxytetradecanoate (B). Diagnostic fragments are: methyl (13,14-$^2H_2$) 11-hydroxytetradecanoate, 215 (M – R_1) and 183 (M – R_1 – CH_4O) and methyl (14,14,14-$^2H_3$) 12-hydroxytetradecanoate, 229 (M – R_1) and 197 (M – R_1 – CH_4O).

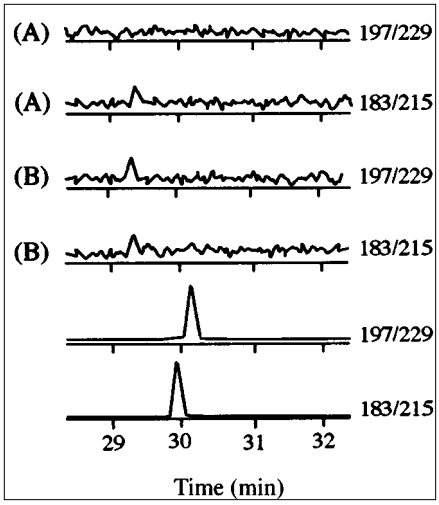

FIG. 3. GC–MS traces of *Spodoptera littoralis* pheromone glands after acid methanolysis with sulfuric acid/methanol/benzene (0.1:4:2, by vol) (90°C/1 h) (A) and 6 N HCl/MeOH (1:2, vol/vol) (90°C/1 h) (B) by monitoring the diagnostic ions for methyl 11-hydroxytetradecanoate (*m/z* 183 and 215) and methyl 12-hydroxytetradecanoate (*m/z* 197 and 229). The two bottom traces correspond to standard methyl (13,14-$^2H_2$) 11-hydroxytetradecanoate (retention time 29.94 min) and methyl (14,14,14-$^2H_3$) 12-hydroxytetradecanoate (retention time 30.17 min), whose mass spectra are shown in Figure 2.

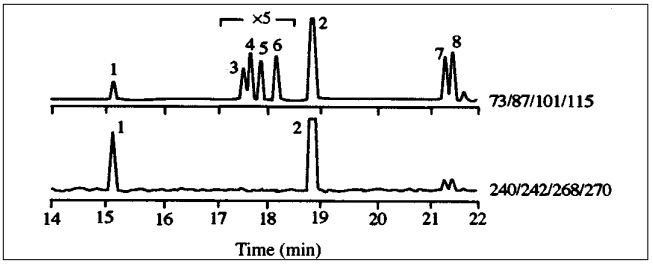

FIG. 4. GC–MS traces of *Spodoptera littoralis* pheromone glands after acid methanolysis with 14% BF_3–MeOH at 4°C for 15 h (A). A trace of unreacted FAME extract (B) is also depicted to show the presence of the monounsaturated FAME. Analyses were performed under SCAN mode, selecting, in each trace, the ions shown at right, which correspond to: 240, methyl (*Z*)-9, (*E*)-11 and (*Z*)-11-tetradecenoate; 242, methyl tetradecanoate; 268, methyl (*Z*)-11-hexadecenoate, 270, methyl hexadecanoate; 73, 12-MeO-14:Me; 87, 11-MeO-14:Me; 101, 10-MeO-14:Me and 12-MeO-16:Me, and 115, 9-MeO-14:Me and 11-MeO-16:Me. Peaks are: 1, methyl tetradecanoate; 2, 9-MeO-14:Me; 3, 10-MeO-14:Me; 4, 11-MeO-14:Me; 5, 12-MeO-14:Me; 6, methyl hexadecanoate; 7, 11-MeO-16:Me; 8, 12-MeO-16:Me; 9, methyl (*Z*)-9-tetradecenoate; 10, methyl (*E*)-11-tetradecenoate; 11, methyl (*Z*)-11-tetradecenoate; and 12, methyl (*Z*)-11-hexadecenoate. Mass spectra of compounds 2 to 5, 7, and 8 are shown in Figure 5.

In light of the above results, showing that *Z*11-14:Me is stable under acidic methanolysis conditions (i) and (ii), both procedures were considered suitable for acidic methanolysis and further reliable analysis of the occurrence of hydroxy FAME in natural extracts.

Authentic samples of methyl (13,14-$^2H_2$) 11-hydroxytetradecanoate and (14,14,14-$^2H_3$) 12-hydroxytetradecanoate, previously synthesized in our laboratory (12), were first analyzed by GC–MS. The mass spectra of both hydroxy derivatives exhibited, besides the base peak at *m/z* 87, ions corresponding to the loss of R_1 from the molecular ion, as well as a characteristic fragment arising from loss of CH_4O from the $M - R_1$ ion (Fig. 2). Natural extracts were then prepared by acidic methanolysis and analyzed by GC–MS, monitoring the characteristic expected ions for both methyl 11-hydroxytetradecanoate (*m/z* 183 and 215) and methyl 12-hydroxytetradecanoate (*m/z* 197 and 229). None of the above procedures gave rise to the expected hydroxy FAME, as concluded from the absence of the corresponding peaks in the GC–MS traces (Fig. 3). The possibility that the hydroxyacyl derivatives, if any, might have decomposed under the methanolysis conditions was ruled out by submitting a sample of methyl (14,14,14-$^2H_3$) 12-hydroxytetradecanoate to the same reactions. Analysis by GC–MS revealed that no product other than the starting hydroxy ester was present in the chromatogram (data not shown). In conclusion, the present results indicate that 11- or 12-hydroxytetradecanoic acids are absent in *S. littoralis* pheromone glands, which, in addition to our previous data of mass-labeling experiments (12), indicates that formation of (*E*)-11-tetradecenoic acid does not involve hydroxylation and further dehydration of tetradecanoic acid, but probably the direct action of an *E*-11 desaturase on this saturated precursor. This possibility is currently being investigated in our laboratories.

On the other hand, transformation of alkenes into methoxy derivatives has been reported as a simple and convenient microanalytical technique for the location of double bonds in alkenes and the further characterization of monounsaturated hydrocarbons (9,10). Those methoxy derivatives have generally been obtained by methoxy mercuration followed by demercuration of the parent olefin (9,10). The result obtained with standard *Z*11-14:Me indicates that reaction of olefins with BF_3–MeOH may be a simple alternative method for derivatization of alkenes to their corresponding methoxy derivatives. This procedure was validated in the location of double bonds of *S. littoralis* sex pheromone biosynthetic precursors. When a lipidic extract was submitted to conditions (iii), GC–MS analyses of the resulting extract showed, in addition to methyl esters derived from the usual fatty acyl intermediates, the presence of low amounts,

TABLE 1
Retention Times and Characteristic Ions of the Methoxy Fatty Acid Methyl Esters Formed from the Monoene Biosynthetic Intermediates of *Spodoptera littoralis*

Compound	Retention times (min)[a]	Diagnostic ions[b]	
		A^+	B^+
9-MeO-14:Me	17.67	115	201
10-MeO-14:Me	17.75	101	215
11-MeO-14:Me	17.89	87	229
12-MeO-14:Me	18.19	73	243
11-MeO-16:Me	21.34	115	229
12-MeO-16:Me	21.44	101	243

[a]See the Experimental Procedures section for GC–MS conditions.
[b]Structure of ions A^+ and B^+ is depicted in Figure 1.
Abbreviations: 9-MeO-14:Me, methyl 9-methoxytetradecanoate; 11-MeO-16:Me, methyl 11-methoxyhexadecanoate, and so on.

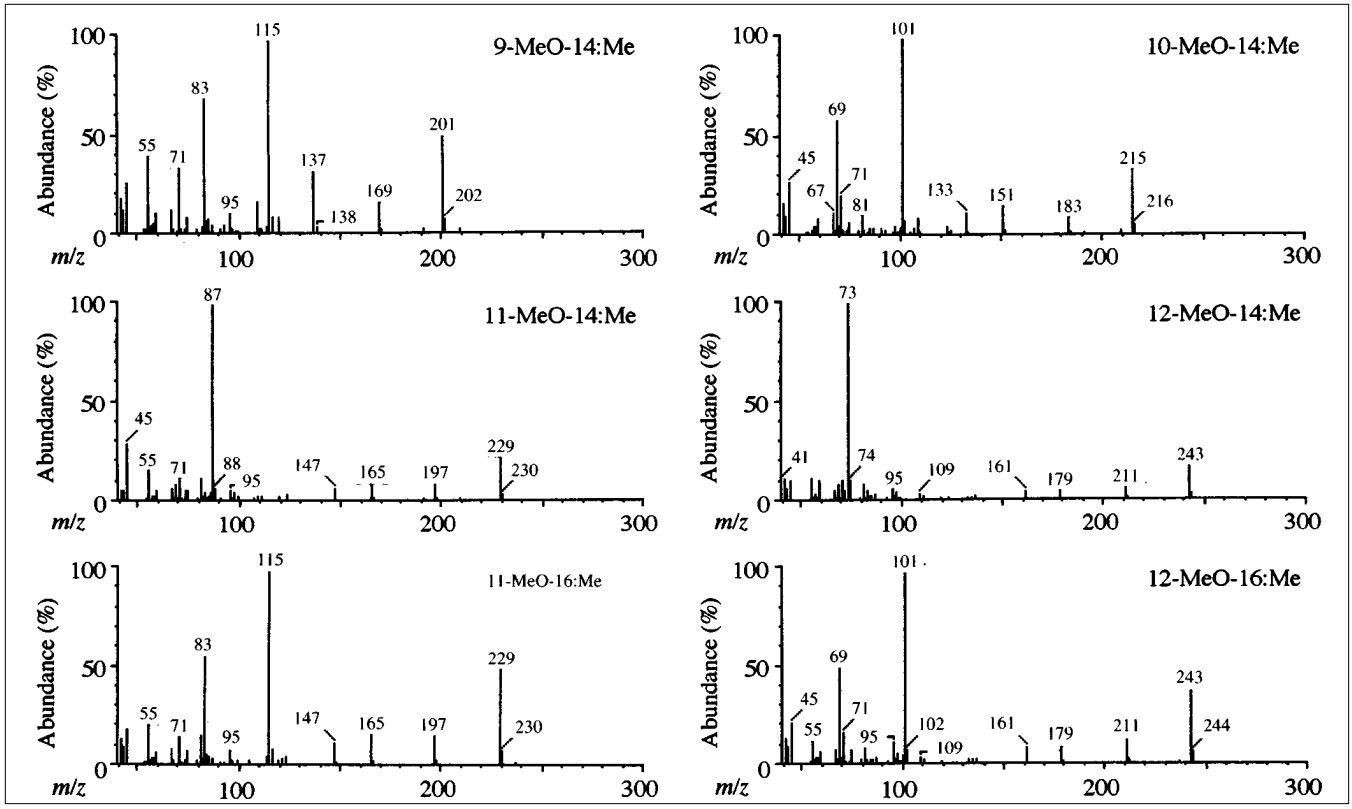

FIG. 5. Mass spectra of the methoxy fatty acid methyl esters found in samples prepared by acid methanolysis with 14% BF_3–MeOH (4°C/15 h) of a lipidic extract of *Spodoptera littoralis* pheromone glands. Mass spectra correspond to peaks 2 (9-MeO-14:Me); 3 (10-MeO-14:Me); 4 (11-MeO-14:Me); 5 (12-MeO-14:Me); 7 (11-MeO-16:Me); and 8 (12-MeO-16:Me) of Figure 4. Diagnostic fragments for each methoxy derivative are indicated in Table 1.

around 10% of the parent monoene, of a mixture of methoxy FAME (Fig. 4, Table 1), including methyl 9-methoxytetradecanoate (9-MeO-14:Me), 10-methoxytetradecanoate (10-MeO-14:Me), 11-methoxytetradecanoate (11-MeO-14:Me), 12-methoxytetradecanoate (12-MeO-14:Me), 11-methoxyhexadecanoate (11-MeO-16:Me) and 12-methoxyhexadecanoate (12-MeO-16:Me). All these compounds exhibited in their mass spectra the two characteristic oxygen-containing fragments A^+ and B^+ (Fig. 5, Table 1) resulting from cleavage between the adjacent methoxy-containing carbons.

Although treatment with BF_3–MeOH at 90°C for 1 h led to partial formation of these adducts, when the methanolysis was carried out at 4°C for 15 h all the monounsaturated FAME were completely transformed into the corresponding methoxy derivatives, as concluded from the absence of monounsaturated FAME in the GC–MS traces (Fig. 4). It is worth mentioning that these methoxy fatty esters were not formed in samples coming from lipidic extracts submitted to acid methanolysis under conditions (i) or (ii). Thus, treatment of lipidic extracts with 14% BF_3–MeOH at 4°C for 15 h is a very simple alternative procedure to obtain methoxy derivatives of monounsaturated fatty acids, which are very useful for the location of the double bond in the parent alkene. Thus, in this case, the detection of 9-, 10-, 11- and 12-MeO-14:Me and 11- and 12-MeO-16:Me in acid-methanolyzed lipidic extracts of *S. littoralis* pheromone glands agrees with the occurrence of the parent C_{14} and C_{16} monounsaturated fatty acyl intermediates previously identified (2) in this species.

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A Human Dietary Arachidonic Acid Supplementation Study Conducted in a Metabolic Research Unit[1]

Arachidonic acid is arguably the single most important fatty acid in the panoply of lipid metabolism. Indeed, its importance in human physiology cannot be overemphasized. Arachidonic acid itself is an important component of phospholipids and cell membranes. It is the unique precursor of most of the eicosanoids with significant biological activity. Although most higher mammals, with perhaps the exception of the cat family, can synthesize arachidonic acid from linoleic acid, so it is not an essential dietary component, it is a normal part of the human diet particularly among those of us who eat animal products. Despite arachidonic acid's acknowledged importance in human metabolism, few human feeding studies using arachidonic acid have been reported. Investigators have exhibited a marked lack of enthusiasm for such studies perhaps because many investigators viewed dietary arachidonic acid as a potentially harmful substance. This supposition was based mainly on the work of Seyberth *et al.* (1) in humans, and other investigators in nonhuman species (2,3). Also, sufficient quantities of arachidonic acid for human feeding studies were not easy to obtain. Still, there was a body of literature that suggested that dietary arachidonic acid was not harmful (4–6).

With the recent availability of an edible oil containing 50% arachidonic acid as mixed triglycerides, we decided to mount a human feeding study in which arachidonic acid would be fed to a group of healthy men under rigidly controlled conditions. The study was conducted at the Agricultural Research Service's Western Human Nutrition Research Center in San Francisco, California, during 1994. It involved the collaboration of investigators from several laboratories and the donation to the United States Department of Agriculture of the arachidonic acid-containing oil (ARASCO®, Martek Biosciences Corp., Columbia, MD). This symposium marks the first presentation of the results gleaned from the mass of data collected during the study. There were six presentations at the symposium covering various aspects of the study—from the effect of arachidonic acid on blood platelets to eicosanoid metabolism, immune response, and intermediate metabolism of arachidonic acid precursors. We have now gathered the presentations as papers and are publishing them here as a unit allowing interested readers to obtain a complete overview of this study in a single place. The principal investigators for this study hope the results justify their efforts and, more importantly, lead to further studies of dietary arachidonic acid in humans.

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[1]This symposium took place at the Annual Meeting of the American Oil Chemists' Society in Indianapolis, Indiana, April 28–May 1, 1996.

A Human Dietary Arachidonic Acid Supplementation Study Conducted in a Metabolic Research Unit: Rationale and Design

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ABSTRACT: While there are many reports of studies that fed arachidonic acid (AA) to animals, there are very few reports of AA feeding to humans under controlled conditions. This 130-d study was conceived as a controlled, symmetrical crossover design with healthy, adult male volunteers. They lived in the metabolic research unit (MRU) of the Western Human Nutrition Research (WHNRC) for the entire study. All food was prepared by the WHNRC kitchen. The basal (low-AA) diet consisted of natural foods (30 en% fat, 15 en% protein, and 55 en% carbohydrate), containing 210 mg/d of AA, and met the recommended daily allowance for all nutrients. The high-AA (intervention) diet was similar except that 1.5 g/d of AA in the form of a triglyceride containing 50% AA replaced an equal amount of high-oleic safflower oil in the basal diet. The subjects (ages 20 to 39) were within −10 to +20% of ideal body weight, nonsmoking, and not allowed alcohol in the MRU. Their exercise level was constant, and their body weights were maintained within 2% of entry level. Subjects were initially fed the low-AA diet for 15 d. On day 16, half of the subjects (group A) were placed on the high-AA diet, and the other group (B) remained on the low-AA diet. On day 65, the two groups switched diets. On day 115, group B returned to the low-AA diet. This design, assuming no carryover effect, allowed us to merge the data from the two groups, with the data comparison days being 65 (low-AA) and 115 (high-AA) for group B and 130 (low-AA) and 65 (high-AA) for group A. The main indices studied were the fatty acid composition of the plasma, red blood cells, platelets, and adipose tissue; *in vitro* platelet aggregation, bleeding times, clotting factors; immune response as measured by delayed hypersensitivity skin tests, cellular proliferation of peripheral blood mononuclear cells in response to various mitogens and antigens, natural killer cell activity, and response to measles/mumps/rubella and influenza vaccines; the metabolic conversion of deuterated linoleic acid to AA and the metabolic fate of deuterated AA in the subjects on and off the high-AA diet; and the production of eicosanoids as measured by excretion of 11-DTXB_2 and PGI_2-M in urine. The results of these studies will be presented in the next five papers from this symposium.
Lipids 32, 415–420 (1997).

Arachidonic acid (AA) is the immediate metabolic precursor of the major biologically active eicosanoids (1,2), such as prostaglandins, thromboxanes, prostacyclins, and leukotrienes as well as a large number of hydroxy eicosatetraenoic acids and their metabolic products. It is also a normal constituent of the human diet (3) if one is not a vegan. Lacto-ovo vegetarians would consume AA from egg yolks. The average amount of AA in Western diets is not certain but has been estimated at between 50 and 300 mg per day (4,5). Jonnalagadda *et al*. (6) reported that the U.S. diet contains approximately 100 mg/d of AA based on the USDA Nationwide Food Consumption Survey for 1987–1988. Sinclair and O'Dea (7) reported that the Australian diet contained between 80 and 100 mg/d of AA. It is likely that general food consumption surveys based on computerized food composition tables underestimate the amount of AA in the daily diet because most food composition data were generated by food analysis that either did not report values for AA or underreported the true amount in the product.

Because of its metabolic importance, there is considerable interest in the metabolic and physiological effects of dietary AA, but most of the data available currently come from animal experiments (8–12). Also, AA has a reputation as being a harmful substance if it is consumed in significant amounts. AA injected as the free acid intravenously in rabbits causes rapid disseminated intravascular coagulation and the death of the animals (13,14). In animal studies, large amounts of AA in the ester form do not seem to cause significant harm to the animals in the short term (8–12,15,16). Seyberth *et al*. (17) reported a human feeding study in which 6 g/d of AA were given to normal male subjects in the form of ethyl ester. Although the study was planned to continue for a month, it was

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Abbreviations: AA, arachidonic acid; FA, fatty acid; MRU, metabolic research unit; SD, stabilization diet.

terminated after 3 wk because of markedly increased *ex vivo* ADP-induced aggregation (10 to 60%) by platelets from the volunteers. Ever since this 1975 report (17), feeding studies using AA in any form in humans have been avoided, presumably out of fear of adverse consequences.

Given the large number of animal feeding studies (8–12, 15,16) demonstrating little or no harmful effects of dietary AA when fed in the ester form, and the fact that even in the study by Seyberth *et al*. (17) there was no actual physical harm to the subjects coupled with the fact that AA is a normal constituent of the human diet, it seems that human feeding studies with AA could be conducted safely. More recently Sinclair and colleagues (18) investigated the effects of dietary AA in Australians using all natural food diets with modestly elevated amounts of AA (7,18) In addition, within the last decade a natural triglyceride produced by selected strains of algae (ARASCO®) that contained 50% AA became commercially available. Thus, because of the physiological and metabolic importance of eicosanoids in humans and the sparse availability of good data from human feeding studies using AA, we decided to mount a human feeding study in which we fed healthy male subjects 1.5 g of AA per day for 50 d. The study was conducted in a metabolic ward setting in which all aspects of the volunteers' lives could be strictly controlled. The major hypothesis to be tested was that dietary AA at this level would be metabolized differently from AA synthesized *in vivo* from linoleic acid and that it has significant influence on platelet function, immune response, and eicosanoid production.

The main physiological and biochemical parameters to be studied were platelet aggregation *in vitro,* blood lipid levels, the fatty acid composition of various tissues, the immune response as measured by response to several antigens, vaccines, and the delayed hypersensitivity skin tests, production of thromboxane and prostacyclin as measured by their metabolic products in urine, and the actual metabolism of dietary fatty acids (FA) as determined by feeding deuterium-labeled FA and measuring the metabolic products in blood over a time course of up to 72 h. This paper will describe the design of the study, the diets and the FA composition of the ARASCO® oil and the actual diets, and the timing of the blood draws. Subsequent papers in this symposium will present the results obtained in the study to date on platelet function, blood lipids, lipoproteins and apoproteins, tissue FA composition, effect on eicosanoid metabolism, immune response, and the metabolism of 20:3n6 in high- and low-AA diets.

STUDY DESIGN

The design was a single blind crossover, feeding study in which healthy volunteers lived in the metabolic unit (MRU) of the Western Human Nutrition Research Center (San Francisco, CA) for 130 d. A schematic of the design is presented in Figure 1. The physical parameters of the 10 male volunteers who completed the study are: age, yr, 31.6 ± 6.7; weight, kg, 72.7 ± 7.7; body mass index, kg/m^2, 23.5 ± 3.6; blood pressure, systolic, 115.9 ± 9.5; blood pressure, diastolic, 73.2 ± 7.8; no smokers. The study had a symmetrical design in which all subjects were fed a stabilization diet for 15 d after they entered the MRU. On day 16 of the study, six of the subjects, designated Group A, were switched to a diet containing 1.7 g/d of AA (210 mg from the basal diet and 1.5 g from ARASCO® oil). The remaining six subjects, designated Group B, continued to consume the basal diet which contained 210 mg of AA. On day 65 of the study, the two groups switched diets. On day 115, the study Group B was returned to the basal diet. The subjects were discharged from the MRU on day 130 of the study.

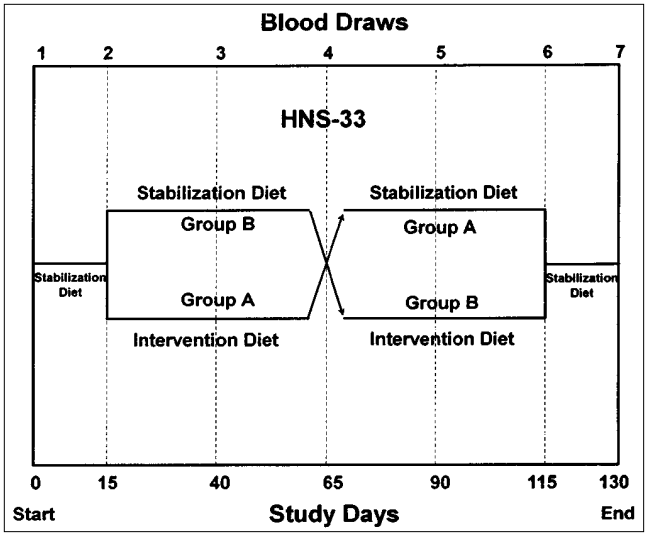

FIG. 1. A schematic plan of the crossover design for this feeding study showing the days of the blood drawn and dietary periods. Note that the values were compared for each group after 50 d on the intervention diet to those obtained after 65 d on the stabilization diet. Thus, the data for group A were comparing the data obtained at day 65 with those obtained at day 130, while in group B the data obtained from day 115 were compared to those obtained on day 65.

This design provided a 50-d intervention period and a 65-d control period for each subject. We assumed that a 65-d washout period for Group A was long enough to eliminate any carryover effect from the high-AA diet. This design allowed the two groups to be merged for statistical purposes, and for most analyses we used an n = 10 (two subjects dropped out of the study prior to completion).

MATERIALS AND METHODS

Source and purity of AA (ARASCO® oil). AA, in the form of a natural triglyceride manufactured and purified from algae (Martek Biosciences Corporation, Columbia, MD), was incorporated into salad dressings and yogurt desserts. The ARASCO® oil produced by the Martek Corporation meets all the requirements for an edible oil for human consumption and contains (wt%) 49.7% AA in the form of mixed triglycerides. The other major FA components of ARASCO® oil are 11.1%

16:0; 10.6% 18:0; 8.7% 18:1n-9; 6.5% 18:2n-6; 3.8% 18:3n-6; 0.9% 20:0; 2.5% 20:2n-6; 1.6% 22:0; and 1.2% 24:0. It is refined and purified by the standard methods used in the edible oil processing industry in the United States. It contains 0.025% α-tocopherol and 0.025% ascorbyl palmitate as antioxidants. The Western Human Nutrition Research Center has conducted human feeding studies with polyunsaturated, long-chain FA for over 10 yr. The oil was kept under nitrogen and at −20°C until it was needed for the food preparation. Open bottles were flushed with N_2 before they were resealed and returned to the freezer. Samples from the ARASCO® oil were taken periodically for analysis of their peroxide values and FA composition. As we fed the AA in a cold yogurt cocktail, it is unlikely that any oxidative degradation took place between the time the food was prepared and when it was eaten.

Subjects. The volunteers were recruited from the West Coast of the United States, and consisted of men between the ages of 20 and 39. Initially 12 volunteers were included in the study, but 2 were unable to complete the protocol. The volunteers were given complete physical examinations. Body weights had to be within −10 to +20% of ideal body weights using the Metropolitan Life Insurance Company tables (medium-frame values from the 1983 edition) (19). Evidence of existing illness or chronic disease was an exclusion criterion. Mild hypertension was not an exclusion criterion, but the group that was recruited tended to have blood pressures slightly below average for men in this age group. Smoking, excessive alcohol consumption, or evidence of narcotic abuse were also exclusionary.

Experimental protocol. All the volunteers were confined to the Nutrition Research Suite of the Western Human Nutrition Research Center for the duration of the study. As the subjects stayed within the confines of the Nutrition Suite except for occasional supervised outings, they had no opportunity to consume any food except that provided by the Center. Thus, compliance with the protocol was, of necessity, 100%. In addition, all food intake was monitored, portions were weighed, and subjects were required to consume everything provided them during their meals. (A rubber spatula was provided to ensure that all food was scraped from the plates and eaten.) Food spills were carefully monitored and recorded, and fluid intake, while *ad libitum,* was also measured precisely.

The protocol for this study was approved by the Institutional Review Boards of the University of California at Davis (Davis, CA) and the United States Department of Agriculture (USDA; Washington, D.C.). A crossover design was used so that the subjects acted as their own controls. The subjects were fed the stabilization diet (SD), containing about 30% of calories as fat, for 15 d. The SD had a target macronutrient composition of 30 en% fat, 15 en% protein, and 55 en% carbohydrate. On day 16 of the study, they were divided randomly into two groups. Group B remained on the SD for 50 d. Group A was placed on a diet in which 1.5 g/d of AA (in the form of a triglyceride containing 50% AA, ARASCO®) was added to the diet for 50 d. After 50 d, day 65 of the study, the groups switched diets for the next 50 d of the study. On day 115 of the study, Group B was returned to the SD. On day 130, all the volunteers were discharged from the study and returned to free living status in the community.

During the confinement period, there were blood draws on study days 2, 16, 40, 65, 90 115, and 130. For most analyses, values from samples obtained on days 65 and 130 for group A and days 65 and 115 for group B were pooled, and the data used for statistical analysis. Data from day 65 for Group A and day 115 for Group B were the end of the AA feeding periods, while data from day 130 for Group A and day 65 for group B were the baseline period.

Diets. The diets consisted of natural foods. No dietary supplements were given. The macronutrient composition of the diets is given in Table 1. (A complete description of the diets, listing all the major and minor nutrients, is available upon request.) A 5-d menu cycle was used throughout the study. Proximate analysis was made on five individual diet composite samples taken from each menu once during the study for both the SD and intervention diets. The results for the seven composite samples were averaged to find the actual composition of the diets. No alcohol was included in these diets. The nutrient composition of the diets was calculated from a computerized nutrient data bank using the data from the *USDA Handbook 8* (20), and adjusted to provide at least the recommended daily allowance for known essential nutrients (21).

Table 2 gives the measured FA composition of both diets. Inspection of Table 2 indicates that all the major FA were very similar except that the level of AA was elevated in the high-AA diet and the level of oleic acid was reduced. The 210 mg of AA found in the SD represents the level of AA found in the natural food used in this study. The theoretical level calculated from *Handbook 8* values was only 150 mg. We believe that this difference is caused by missing values and inaccurate analyses in *Handbook 8*. At the beginning of the

TABLE 1
Composition of Diets (% of total calories), Proximal Analysis[a]

	Intervention diet (high-AA)		Stabilization diet (low-AA)	
	Measured	Target value	Measured	Target value
Macronutrient energy distribution				
Protein	15.2	15.0	15.8	15.0
Fat	27.9	30.0	27.1	30.0
Carbohydrate	56.9	55.0	57.1	55.0
Cholesterol content, mean, mg/d	—	360	—	360
Fatty acid energy distribution				
Saturated	7.7	10	7.4	10
Monounsaturated	9.7	10	10.3	10
Polyunsaturated	7.7	10	7.0	10
P/S Ratio	1.0	1.0	0.9	1.0

[a]AA, arachidonic acid; P/S, polyunsaturated/saturated.

TABLE 2
Fatty Acid Composition (wt%) of Experimental Diets[a]

Fatty acid methyl esters	Intervention diet (high-AA)	Stabilization diet (low-AA)	*P* values
12:0 (laurate)	0.8 ± 0.4	0.5 ± 0.3	
14:0 (myristate)	2.5 ± 1.3	2.3 ± 1.3	
15:0	0.3 ± 0.1	0.3 ± 0.1	
16:0 (palmitate)	15.5 ± 3.2	15.6 ± 3.4	
16:1n-9	0.8 ± 0.3	0.8 ± 0.3	
17:0	0.3 ± 0.1	0.3 ± 0.1	
18:1 DMA[b]	0.2 ± 0.1	0.2 ± 0.1	
18:0 (stearate)	7.9 ± 1.5	7.9 ± 1.7	
18:1*t*, all isomers	6.3 ± 1.9	6.4 ± 1.9	
18:1n-9 (oleate)	30.7 ± 2.6	33.8 ± 2.8	
18:1n-7	1.5 ± 0.2	1.6 ± 0.2	
18:1n-5	1.5 ± 0.6	1.5 ± 0.7	
18:2*tt* and 19:0	0.6 ± 0.2	0.5 ± 0.2	
18:2n-6 (linoleate)	23.8 ± 4.1	23.8 ± 4.5	
18:3n-6	0.4 ± 0.1	0.4 ± 0.0	
18:3n-3 (α-linolenate)	1.9 ± 0.7	1.8 ± 0.5	
20:1n-11	0.4 ± 0.1	0.3 ± 0.0	
22:0 (behenate)	0.3 ± 0.0	0.3 ± 0.0	
20:4n-6 (arachidonate)	2.1 ± 0.1	0.2 ± 0.1	$P < 0.001$
Total	97.7 ± 0.3	98.5 ± 0.4	
Unknowns	2.3 ± 0.3	1.5 ± 0.4	

[a]Means ± SD ($n = 5$). See Table 1 for abbreviation.
[b]DMA, dimethylacetal.

study, each participant's caloric intake was estimated, and the caloric content of the meals calculated to maintain current body weight. As the estimates were subject to error, the participants were weighed every day, and the caloric value of their meals adjusted occasionally, if necessary, to maintain their weight within the limits of the study. Thus, the average weights of the participants did not vary significantly during the 130 d of the study. If all the intakes for each volunteer were averaged during the entire study, the mean energy intake was approximately 2800 Kcal/d.

Measurements. Blood was drawn between 7:00 and 8:00 a.m. after an overnight fast of at least 12 h. Blood was drawn into vacutainers with appropriate anticoagulants, using a Teflon catheter (Angiocath; Deseret Medical, Sandy, UT) for FA analysis, or syringes containing 1 mL of citrate solution for platelet studies.

Studies with deuterated AA. The first experiment involved deuterated AA fed to three subjects eating high-AA diet. A second group of three subjects eating the low-AA diet also received the deuterated AA. An equal amount of deuterated oleic acid was fed at the same time. Blood samples were drawn immediately before feeding the tracers and at 4, 6, 8, 12, 24, 48, and 72 h. A second experiment using different subjects (three from each diet group) from this study fed deuterated 18:2n-6 to subjects pre-fed the diets described in the first experiment above. Details of this studies are found in the paper by Emken *et al.* (22).

Physiological parameters examined. We determined the blood lipid concentrations, FA composition of the tissue samples, eicosanoid levels in urine, blood, and media from cultured cells. Bleeding times *in vivo* and platelet function *in vitro* were determined. Deposition of AA in the adipose tissue was followed using tissue biopsies. The volunteers' immune status was evaluated by determining the number of lymphocytes and their subsets, monocytes and granulocytes, lymphocyte proliferation in response to T and B cell mitogens, *in vitro* production of IL-1, IL-2, IL-2R, IL-6, and TNF and by determining their concentration through ELISA assays. Other indices of immune response tested included natural killer cell activity, delayed hypersensitivity skin response to a battery of seven recall antigens, and primary and secondary antibody responses to viral and bacterial antigens (23,24).

RESULTS

In this study we could detect very few physiological effects from dietary AA when consumed at the level of 1.7 g/d for 50 d. Plasma PL and cholesterol esters showed a marked increase in their content of AA, while the plasma triglyceride level exhibited only minimal incorporation of AA. Adipose tissue did not incorporate any significant amount of AA during this study. Blood lipid levels showed no changes nor did the apoproteins and lipoproteins. Perhaps most importantly we detected no changes in platelet aggregation or bleeding times contrary to the report by Seyberth *et al*. (17). Most indices of the immune response were not affected either. The detailed results to date from this study are presented in the next five papers from this symposium.

DISCUSSION

For several decades now polyunsaturated FA have been considered beneficial compared to saturated FA in the diet (25–27). This was particularly true between 1950 and 1970. Then, with greater knowledge of the role of eicosanoids in human physiology and the rise in interest in n-3 polyunsaturated FA, some investigators (28–30) questioned the wisdom of raising the level of n-6 FA in the diet above that commonly consumed in a Western diet. AA in particular was considered an undesirable dietary constituent, particularly as it was known to have adverse effects in animals (13,14), coupled with the report of Seyberth *et al.* (17) that indicated that AA in the human diet increased the tendency for platelets to aggregate. Conversely, more recent work in infant nutrition has suggested that newborn infants require dietary AA for optimal growth and development (31,32). Still, very little work has been reported in humans on AA feeding studies since the report of Seyberth *et al*. (17) in 1975. Sinclair and colleagues (18,33,34) have reported some studies on AA in human diets from Australia with selected food that increases the level of AA in the diet, although well below the levels used in the study by Seyberth *et al*. (17). Sinclair and colleagues (18,33,34) observed no adverse effects from their AA-enhanced diets.

Of course, none of these negative results from this study should be taken to mean that dietary AA has no metabolic in-

fluences in humans. We did detect significant changes in the production of urinary eicosanoids. In addition, we (22) found changes in the metabolism of the polyunsaturated FA in the presence of elevated dietary AA. Furthermore, while the amount of AA fed in this study was 5 to 10 times that in the typical Western diet (6,35), it was still well below that used in the study reported by Seyberth *et al*. (17). We cannot predict whether higher levels of dietary AA, or longer feeding period, will have major metabolic and physiological consequences. However, it is interesting that in 1961 Kingsburg *et al.* (36) fed 10 g/d of ethyl AA to two students for 11 d without any reported ill effects. The results from this study suggest that dietary AA fed at 1.5 g/d is not harmful to humans nor does it affect blood lipid levels [Kingsburg *et al.* (36) reported a cholesterol-lowering effect from 10 g of ethyl arachidonate] or platelet function while only having minimal effects on the normal immune response. Perhaps the most important implication from this work is the suggestion that the physiological control mechanisms for FA homeostasis in humans shunt dietary AA into specific metabolic pathways. These pathways then tightly control AA utilization and conversion to other compounds in the body. Whether these control mechanisms can be overwhelmed by increasing the amount of AA in the diet remains to be determined. The detailed results for this collaborative study effort are presented in the five other arachidonic acid symposium papers (22, 37–40).

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The Effect of Dietary Arachidonic Acid on Platelet Function, Platelet Fatty Acid Composition, and Blood Coagulation in Humans

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ABSTRACT: Arachidonic acid (AA) is the precursor of thromboxane and prostacyclin, two of the most active compounds related to platelet function. The effect of dietary AA on platelet function in humans is not understood although a previous study suggested dietary AA might have adverse physiological consequences on platelet function. Here normal healthy male volunteers (n = 10) were fed diets containing 1.7 g/d of AA for 50 d. The control diet contained 210 mg/d of AA. Platelet aggregation in the platelet-rich plasma was determined using ADP, collagen, and AA. No statistical differences could be detected between the aggregation before and after consuming the high-AA diet. The prothrombin time, partial thromboplastin time, and the antithrombin III levels in the subjects were determined also. There were no statistically significant differences in these three parameters when the values were compared before and after they consumed the high-AA diet. The *in vivo* bleeding times also did not show a significant difference before and after the subjects consumed the high-AA diet. Platelets exhibited only small changes in their AA content during the AA feeding period. The results from this study on blood clotting parameters and *in vitro* platelet aggregation suggest that adding 1.5 g/d of dietary AA for 50 d to a typical Western diet containing about 200 mg of AA produces no observable physiological changes in blood coagulation and thrombotic tendencies in healthy, adult males compared to the unsupplemented diet. Thus, moderate intakes of foods high in AA have few effects on blood coagulation, platelet function, or platelet fatty acid composition.
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Although arachidonic acid (AA) is a normal constituent of the human diet (1) if one is not a vegan, it has been suggested that higher levels of AA in the diet may have adverse consequences (2). In 1975 Seyberth *et al.* (3) reported a human feeding study in which 6 g/d of AA were given to healthy male subjects in the form of the ethyl ester. Although the study was planned to continue for longer period, it was terminated after 3 wk because of marked increase in *in vitro* platelet aggregation (10 to 60%) in response to ADP in samples taken from the volunteers.

Despite the caveat introduced into human feeding studies with AA by the Seyberth *et al.* (3) report, there is considerable interest in the metabolic and physiological effects of dietary AA, although most of the data available currently come from animal experiments (4–9). AA injected as the free acid intravenously in rabbits causes rapid disseminated intravascular coagulation and the death of the animals (10,11). In animal studies, large amounts of AA in the ester form do not seem to cause significant harm to the animals in the short term (4–9,12).

Given the large number of animal feeding studies (4–9,12) demonstrating little or no harmful effects of dietary AA when fed in the ester form, and the fact that even in the study by Seyberth *et al.* (3) there was no actual physical harm to the subjects coupled with the fact that AA is a normal constituent of the human diet, it seems that human feeding studies with AA could be conducted safely. In addition, within the last decade a natural triglyceride produced by selected strains of algae (trademarked ARASCO®; Martek Biosciences Corporation, Columbia, MD) that contained 50% AA became commercially available. Thus, because of the physiological and metabolic importance of eicosanoids in humans and the sparse availability of good data from human feeding studies using AA, we decided to mount a human feeding study in which we fed healthy male subjects 1.5 g of AA per day for 50 d. The study was conducted in a metabolic ward setting in which all aspects of the volunteers life could be strictly controlled.

STUDY DESIGN

The study design has been described in the previous paper in this series (13) The design was a single-blind cross-over feeding study in which 12 healthy volunteers lived in the metabolic unit (MRU) of the Western Human Nutrition Research Center (San Francisco, CA) for 130 d. The study had a symmetrical design in which all subjects were fed a stabilization diet for 15 d after they entered the MRU. On day 16 of the study, six of the subjects, designated Group A, were switched

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Abbreviations: AA, arachidonic acid; FA, fatty acid; MRU, metabolic research unit; PRP, platelet-rich plasma.

to a diet containing 1.7 g/d of AA—210 mg from the basal diet and 1.5 g from ARASCO® oil). The remaining six subjects, designated Group B, continued to consume the basal diet which contained 210 mg of AA. On day 65 of the study the two groups switched diets. On day 115 of the study Group B was returned to the basal diet. The subjects were discharged from the MRU on day 130 of the study.

MATERIALS AND METHODS

A description of the oil (ARASCO®) and its fatty acid (FA) composition are given in the previous paper (13). The oil was kept under nitrogen and at −20°C until it was needed for the food preparation. Open bottles were flushed with N_2 before they were resealed and returned to the freezer. As we fed the AA in a cold yogurt cocktail, it is unlikely that any oxidative degradation took place between the time the food was prepared and when it was eaten.

Subjects. The physical characteristics of the subjects and the recruitment process were described in the previous paper (13). Initially, 12 volunteers were included in the study, but two were unable to complete the protocol.

Experimental protocol. Details of the experimental protocol were given in the previous paper (13). The protocol for this study was approved by the Institutional Review Boards of the University of California at Davis and the United States Department of Agriculture (USDA), Washington, D.C.). A crossover design was used so that the subjects acted as their own controls. The subjects were fed the stabilization diet, containing about 30% of calories as fat for 15 d and then divided into two groups and fed either the stabilization diet or the high-AA diet for 50 d, at which time (study day 65) the two groups were crossed over for the remainder of the study.

During the confinement period, blood was drawn on study days 2, 16, 40, 65, 90 115, and 130. For most analyses, values from samples obtained on days 65 and 130 for group A and days 65 and 115 for group B were pooled and the data used for statistical analysis. Data from day 65 for Group A and day 115 for Group B were the end of the AA feeding periods, while data from day 130 for Group A and day 65 for group B were the baseline period.

Diets. The nutrient composition of the diets was calculated from a computerized nutrient data bank using the data from *USDA Handbook* (8). *The Composition of Foods* (14) and adjusted to provide at least the recommended daily allowance for known essential nutrients (15). A complete description of the diet is given in the previous paper (13). A complete description of the diets, listing all the major and minor nutrients, is available upon request.

Measurements. Blood was drawn according to the schedule shown in Figure 1 of Reference 13 between 7:00 and 8:00 a.m. after an overnight fast. It was drawn into vacutainers with appropriate anticoagulants using a Teflon catheter, through a 20-gauge needle (Angiocath; Deseret Medical, Sandy, UT) for FA analysis, or syringes containing 1 mL of citrate solution for platelet aggregation studies. Platelet-rich plasma (PRP) was prepared by low-speed centrifugation at 4°C for 10 min at $100 \times g$. Platelet-poor plasma was prepared by respinning the blood at 4°C after removal of the PRP for 15 min at $400 \times g$. Red cells for FA analysis were prepared from the residue by resuspending the cells in 10 mL of phosphate-buffered saline and recentrifuging the cells at $400 \times g$ for 15 min three times. The supernatant was discarded. The red cells were frozen at −70°C until their lipids could be extracted.

Platelet aggregation. Platelet aggregation tests were performed as previously described (16). Briefly, solutions of the aggregating agents were prepared as recommended by Chrono-log (Chrono-log Corp., Havertown, PA) as follows: ADP, 1 μM/μL; collagen, 0.5 μg/mL; AA, 0.05 μM. The *in vitro* aggregation characteristics of the subjects' platelets were measured in a Chrono-log Model 560 (Chrono-log Corp., Havertown, PA) dual channel aggregometer. Briefly, 0.45 mL of PRP was added to an aggregation tube containing a Teflon-coated stirring bar. The blank was 0.5 mL of the individual platelet-poor plasma, unstirred. The instrument was zeroed to 10% transmission of the sample vs. the blank. An appropriate amount of aggregating reagent was added to the PRP and aggregation was measured as the increase in transmission of light through the PRP. Generally the sample was monitored until the curve became flat or with some reagents, notably ADP, disaggregation occurred. The threshold for aggregation was defined when the amount of a particular reagent produced approximately 50% of maximum aggregation within 15 min of its addition to the PRP. All aggregation tests were performed within 4 h of the initial blood draw with PRP that was kept a 0°C until warmed to 37°C for the aggregation test.

Platelets counts. Platelet counts were determined with an electronic particle counter, a Baker-Serono Model 9000 Differential Cell Counter (Allentown, PA) electronic particle counter and cytometer using the manufacturer's recommended procedures (17).

Bleeding time. Bleeding times were measured between 10 and 11 a.m. on the left or right proximal forearm of each subject using a 1-mm deep by 1-cm long incision using a Simplate II bleeding time device (Organon Teknika, Durham, NC). A wipe of the incision was made every 30 s. Time was measured with a hand-held stop watch.

Clotting factors. Prothrombin time and the partial thromboplastin times were measured by the methods described by Houghie (18). Briefly platelet-poor plasma was prepared as described and the prothrombin time and partial thromboplastin time were measured using the Bio/Data Corporation Model MCA 110 instrument (Bio/Data Corporation, Hatboro, PA) with Organon Teknika coagulation reagents. All tests for the clotting factors were performed within 1 h of the separation of the plasma from the whole blood. Plasma samples were kept on ice until warmed to 37°C for the test.

Lipid extraction and FA analysis. Platelets were extracted by the procedures described by Nelson (19,20) using chloroform/methanol (2:1, vol/vol). Samples were then transmethy-

lated with methanolic HCl (7%, w/w) by the procedures described elsewhere (21,22). When the total lipid extract was transmethylated, the impurities, extracted into the hexane phase after termination of the reaction, were removed by thin-layer chromatography as described by Nelson (20). Because the amount of material available from human platelets for FA analysis was limited, FA methyl esters were obtained by direct transmethylation of the intact tissue without prior solvent extraction as described in an earlier publication (23). The conditions of the capillary gas–liquid chromatography have been described previously (21). Briefly, the column was a 30-m by 0.025-mm fused quartz column coated with SP-2340 purchased from Supelco, Inc. (Bellefonte, PA). The gas–liquid chromatography data were processed with Hewlett-Packard (Palo Alto, CA) ChemStation software running on an IBM-compatible desktop computer.

Statistical analysis. The clotting data and aggregation data were analyzed using the paired T-test with an $n = 10$ or analysis of variance with repeated measures. Analysis of variance was used to determine if Group A and Group B data were comparable. The FA data were analyzed with a single-tailed T-test. All the statistical analyses were performed with the PC SAS data system, version 6.11, obtained under license from the SAS Institute (Chapel Hill, NC, 1993).

RESULTS

In this study we could detect very few physiological changes in the blood clotting systems, platelet function, or the platelet FA composition in our subjects resulting from increased levels of dietary AA when consumed at the level of 1.7 g/d for 50 d. The average bleeding times for the 10 subjects after consuming the high-AA diet for 50 d were 3.94 and 3.56 min after they had consumed the low-AA diet, respectively. The difference between these values did not reach statistical significance ($P = 0.06$).

Table 1 lists the platelet aggregation *in vitro* and the platelet counts determined after 50 d on high-AA diets and 65 d on the low-AA diet for the 10 subjects in this study. Again there were no statistically significant changes in either the aggregation values or the platelet counts when the results from the two diets are compared. Table 2 lists some values for the soluble blood coagulation parameters for these subjects after the high-AA or the low-AA diets. The prothrombin time was significantly lower ($P < 0.03$) after the subjects consumed the high-AA diet for 50 d than when they consumed the low-AA diet for 65 d. Neither the partial thromboplastin time nor antithrombin-III values were different after the subjects consumed either diet.

TABLE 1
Platelet Aggregation and Cell Count[a]

	High-AA diet	Low-AA diet	*P* value
Agonist			
ADP (μM)	0.20 ± 0.1	0.17 ± 0.11	0.31
Collagen (μ/mL)	2.33 ± 0.96	2.64 ± 2.08	0.43
AA (mM)	0.40 ± 0.19	0.39 ± 0.11	0.40
Count (1000/mm^3)	339 ± 52	357 ± 60	0.15

[a]($n = 10$); AA, arachidonic acid; mean ± SD.

TABLE 2
Blood Coagulation Properties[a]

Parameter	High-AA diet	Low-AA diet	*P* value
Prothrombin time (s)	11.22 ± 0.44	12.48 ± 0.73	0.03
Partial thromboplastin time, (s)	28.08 ± 2.69	28.45 ± 3.13	0.33
AntiThrombin-III (%)	83.76 ± 19.18	91.32 ± 22.12	0.23

[a]($n = 10$). See Table 1 for abbreviation; mean ± SD.

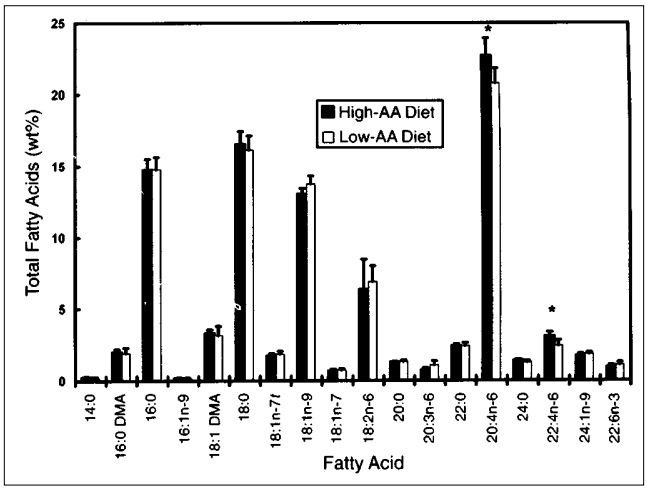

FIG. 1. The total fatty acid composition of the platelets after the consumption of the low-arachidonic acid (AA) and high-AA diets by the subjects in this study. Individual fatty acids are designated by carbon chain length/number of double bonds in the molecule and the position of the first double bond from the methylene end of the molecule. The asterisks designate fatty acids that are significantly different between the two diet groups with P values <0.05. Only fatty acids present in amounts greater than 0.5% of the total fatty acids are displayed in this figure; *t, trans* fatty acid; DMA, dimethylacetal.

Figure 1 compares the total FA composition of the whole platelets after the subjects consumed the high-AA diet for 50 d or the low-AA diet for 65 d. Only the AA and 22:4n-6 content of the platelets showed statistically significant changes between the two diets. The percentages of both of these FA increased in the platelets of the subjects after they had consumed the high-AA diets for 50 d when compared to their platelet FA composition on the low-AA diet for 65 d. This is normalized weight percentage data, and if the percentage of one or more FA increases then, obviously, one or more FA must decrease. The percentage of stearic, oleic, and linoleic acids in the platelets did decrease slightly after the subjects consumed the high-AA diet, but the changes did not reach statistical significance, probably because of the relatively small changes and the size of the standard deviations in these measurements.

DISCUSSION

Dietary AA has been consider a potentially hazardous substance to feed to humans for over two decades, particularly if fed in amounts exceeding those found in typical human diets. This presumption was based largely on the study published by Seyberth *et al.* (3) that showed a marked decrease in *in vitro* platelet aggregation times in healthy men consuming 6 g/d of pure ethyl arachidonate. This information, coupled with the supposition that increased production of eicosanoids, as postulated by Lands and colleagues (2,24) and others (25), would have major adverse effects on human health, has discouraged investigators from human feeding studies with AA. During this same period, several animal studies have been published that did not indicate adverse consequences from the consumption of AA (4–9,12).

As our knowledge of FA metabolism and effects of dietary FA has expanded, it became apparent that dietary FA often had metabolic effects and physiological consequences different from endogenously synthesized FA (26,27). Of course, dietary AA is not essential for human health, provided that one's diet contains adequate amounts of linoleic acid. Indeed, it is likely that linoleic acid has essential roles in human physiology beyond being simply a precursor of AA (28–30). If one wanted to investigate AA metabolism and physiological effects, it was safer to feed linoleic acid rather than AA. However, investigators were left with the paradoxical concept that dietary AA was harmless when fed to animal at levels well above those found in nature, but harmful to humans at levels well below those fed to animals. This conflicting information led us to believe that the metabolic effects of dietary AA in humans should be reinvestigated under controlled condition.

The *in vitro* platelet aggregation characteristics were not different in these subjects after they consumed the high-AA diet, nor was the *in vivo* bleeding times. Contrary to our expectations, the mean bleeding times were slightly longer after the subjects had consumed the high-AA diet compared to the value determined after they had consumed low-AA diet although the two values were not statistically different. In addition, our platelet aggregation results conflict with those presented by Seyberth *et al.* (3), who reported a marked shortening of the *in vitro* platelet aggregation after 3 wk of supplementation with 6 g/d of ethyl arachidonate. Here we fed only 1.5 g/d and in the form of a natural triglyceride, containing about 40% AA, mixed with natural foods. Thus, there are several factors that may account for the differences found between this study and the study by Seyberth *et al.* (3). Our conclusion is simply that at the level of AA used in our study AA does not affect platelet aggregation or the bleeding time *in vivo*.

One significant change we noted when the subjects consumed the high-AA diet for 50 d was that their prothrombin time decreased slightly but statistically significantly. Still, both the values observed when the subjects consumed the low-AA diet for 65 d or high-AA diet for 50 d were well within the normal physiological range reported for this coagulation parameter. Thus, there may be no physiological significance to this difference, particularly considering that the antithrombin-III levels were unaffected by the high-AA diet. Antithrombin-III is considered a measure of the tendency for the blood to undergo thrombosis *in vivo* and has been correlated with increased incident of myocardial infraction in humans (31–34).

In this study we increased the amount of AA consumed daily by a factor of eight. This increase had little effect on the level of platelet AA after 50 d on the high-AA diet compared to the AA levels in the platelets when the subjects consumed the low-AA diet. The AA level went from 21 to 24% when the subjects consumed the high-AA diet, but this is hardly an eight fold increase. Platelets normally contain rather large quantities of AA compared with most other tissues (14,35–37). Some hypotheses that could explain this lack of change are: (i) platelets are saturated with AA and cannot absorb any more of this substance; (ii) because of AA's importance in platelet function, its content in platelets is under very strict control; or (iii) the total amount of AA fed during the study (75 g in 50 d) was not enough to significantly influence the body pools of AA that control the platelet FA composition.

If the first hypothesis is correct, it would suggest that the acyl transferases that control the FA composition of the phospholipids in the cell membranes are in equilibrium with the amount of AA normally present and cannot increase the level in platelets further. It may be possible to shift this equilibrium with higher levels of AA in the diet and further increase the percentage of AA in platelets beyond that observed here. Also, it may be that the ratio of AA to other FA, particularly the n-3 FA, may be the most important factor controlling the FA composition of platelets *in vivo*. The argument against the second hypothesis is that when high levels of n-3 FA are incorporated into human diets they are rapidly incorporated into the platelets, causing a reduction in the AA levels and also in the linoleic and oleic acid levels in the platelets. Another experiment, with longer feeding periods or larger amounts of AA, would be needed to test the third hypothesis. We can conclude, however, that the level of AA in platelets is unlikely to be altered much by the variations encountered in AA intake in the typical Western diet.

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The Effect of Dietary Arachidonic Acid on Plasma Lipoprotein Distributions, Apoproteins, Blood Lipid Levels, and Tissue Fatty Acid Composition in Humans

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ABSTRACT: Normal healthy male volunteers (n = 10) were fed diets (high-AA) containing 1.7 g/d of arachidonic acid (AA) for 50 d. The control (low-AA) diet contained 210 mg/d of AA. Dietary AA had no statistically significant effect on the blood cholesterol levels, lipoprotein distribution, or apoprotein levels. Adipose tissue fatty acid composition was not influenced by AA feeding. The plasma total fatty acid composition was markedly enriched in AA after 50 d ($P < 0.005$). The fatty acid composition of plasma lipid fractions, cholesterol esters, triglycerides, free fatty acids, and phospholipid (PL) showed marked differences in the degree of enrichment in AA. The PL plasma fraction from the subjects consuming the low-AA diet contained 10.3% AA while the subjects who consumed the high-AA diet had plasma PL fractions containing 19.0% AA. The level of 22:4n-6 also was different (0.67 to 1.06%) in the plasma PL fraction after 50 d of AA feeding. After consuming the high-AA diet, the total red blood cell fatty acid composition was significantly enriched in AA which mainly replaced linoleic acid. These results indicate that dietary AA is incorporated into tissue lipids, but selectively into different tissues and lipid classes. Perhaps more importantly, the results demonstrate that dietary AA does not alter blood lipids or lipoprotein levels or have obvious adverse health effects at this level and duration of feeding.
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Arachidonic acid (AA) is a normal constituent of the human diet (1) if one is not a vegan, and is found in all cells and tissues in significant amounts (2–4). Human studies using AA have been limited, probably because of suggestions in the literature that higher levels of AA in the diet may have adverse consequences (5,6). Nevertheless, there have been many feeding studies in animals using high levels of AA that have not shown any major adverse effects (7–10). Furthermore, several human studies (5,11–13) have not resulted in any actual harm to the subjects despite some potential adverse changes in a few physiological parameters such as platelet aggregation *in vitro* (5).

Because of its major role in human physiology, there is much interest in the metabolism and physiological function of dietary AA (14,15). In view of the animal studies, the lack of actual adverse consequence of dietary AA in humans, and the commercial availability of a natural triglyceride (TG) containing 50% AA, we decided to conduct a human feeding study with AA in a metabolic research unit (MRU). The details, protocol, and design of the study have been described in a previous paper (16).

Here we report the effects on several lipid parameters of supplementing a natural food diet (containing 210 mg/d AA) with 1.5 g/d of AA in the form of a TG. The supplementation period was 50 d. We examined the blood lipid levels; fatty acid (FA) composition of the plasma, red cells, and adipose tissue (AT); lipoprotein cholesterol levels, and some apoprotein levels.

STUDY DESIGN

The study design was described in detail in the previous paper in this series (16). The design was a single-blind cross-over feeding study in which 12 healthy volunteers lived in the MRU of the Western Human Nutrition Research Center (San Francisco, CA) for 130 d. The study had a symmetrical design in which all subjects were fed a stabilization diet for 15 d after they entered the MRU. On day 16 of the study, six of the subjects, designated Group A, were switched to a diet containing 1.7 g/d of AA (210 mg from the basal diet and 1.5 g from ARASCO® oil; Martek Biosciences Corporation, Columbia, MD). The remaining six subjects, designated Group B, continued to consume the basal diet which contained 210 mg of AA. On day 65 of the study, the two groups switched diets. On day 115 of the study, Group B was returned to the basal diet. Two subjects did not complete the study. The re-

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Abbreviations: AA, arachidonic acid; AT, adipose tissue; FA, fatty acid; FFA, free fatty acids; HDL, high density lipoprotein; LDL, low density lipoprotein; MRU, metabolic research unit; PL, phospholipids; RBC, red blood cells; TG, triglyceride.

maining subjects were discharged from the MRU on day 130 of the study.

MATERIALS AND METHODS

A description of the oil (ARASCO®) and its FA composition is given in the previous paper (16). The oil was kept under nitrogen and at −20°C until it was needed for food preparation. Open bottles were flushed with N_2 before they were resealed and returned to the freezer. As we fed the AA in a cold yogurt cocktail, it is unlikely that any oxidative degradation took place between the time the food was prepared and when it was eaten.

Subjects. The physical characteristics of the subjects and the recruitment process were described in the previous paper (16). Initially, 12 volunteers were included in the study, but two were unable to complete the protocol.

Experimental protocol. Details of the experimental protocol were given in the previous paper (16). The protocol for this study was approved by the Institutional Review Boards of the University of California at Davis and the United States Department of Agriculture (USDA, Washington, D.C.). A crossover design was used so that the subjects acted as their own controls. The subjects were fed the stabilization diet, containing about 30% of calories as fat for 15 d and then divided into two groups and fed either the stabilization diet or the high AA diet for 50 d, at which time (study day 65) the two groups were crossed over for the remainder of the study.

During the confinement period, blood was drawn on study days 2, 16, 40, 65, 90 115, and 130. Although analysis of the various parameters under study was performed on all volunteers on these study days, for most analyses, values from samples obtained on days 65 and 130 for group A and days 65 and 115 for group B were pooled and the data used for statistical analysis. Data from day 65 for Group A and day 115 for Group B were the end of the AA feeding periods, while data from day 130 for Group A and day 65 for group B were the baseline period. The samples from study days 2 and 16 showed no significant differences between groups A and B.

Diets. The nutrient composition of the diets was calculated from a computerized nutrient data bank using the data from *USDA Handbook 8, The Composition of Foods* (17), and adjusted to provide at least the recommended daily allowance for known essential nutrients (18). A detailed description of the low-AA and high-AA diets is given in the previous paper (16). A complete description of the diets, listing all the major and minor nutrients, is available upon request.

Measurements. Blood was drawn according to the schedule described previously (16) between 7:00 and 8:00 a.m. after an overnight fast. It was drawn into vacutainers with appropriate anticoagulants using a Teflon catheter (Angiocath; Deseret Medical, Sandy, UT) for fatty acid analysis. Red cells for FA analysis were prepared from the residue by resuspending the cells in 10 mL of phosphate-buffered saline and recentrifuging the cells at $400 \times g$ for 15 min three times. The supernatant was discarded. The red cells were frozen at −70°C until their lipids could be extracted. AT biopsies were taken from the buttocks using a hypodermic syringe equipped with 13-gauge stainless-steel needles (19). The samples (10 to 50 mg) were immediately transferred to cold (0°C) isotonic saline. The samples were transferred within an hour to a −70°C freezer and stored until further processing.

Lipid extraction and FA analysis. Plasma and red cell samples were extracted by the procedures described by Nelson (20,21) using chloroform/methanol (2:1, vol/vol). Samples were then transmethylated with methanolic HCl (7%, w/w) by the procedures described previously (22,23). When the total lipid extract was transmethylated, the impurities, extracted into the hexane phase after termination of the reaction, were removed by thin-layer chromatography as described elsewhere (21). The conditions of the capillary gas–liquid chromatography have been described previously (22). Briefly, the column was a 30 m by 0.025 mm fused quartz column coated with SP-2340 purchased from Supelco, Inc. (Bellefonte, PA). The gas–liquid chromatography data were processed with Hewlett-Packard ChemStation software running on an IBM-compatible desktop computer.

Apoprotein analysis. The apoproteins AI and B were analyzed in the Laboratory of Dr. Ernst Schaefer of the USDA Center on Nutrition and Aging, Tufts University Medical Center (Boston, MA). The plasma apolipoproteins, ApoA-I and ApoB, were measured by a turbidimetric procedure using a Spectrum CCX analyzer (Abbott Diagnostics with reagents and calibrators from INCSTAR (Stillwater, MN) (24, 25). The Lp(a) was determined in collaboration with Dr. Steven Silbermann of Perlimmune, Inc. (Rockville, MD), using an ELISA technique developed in Dr. Silbermann's laboratory (26,27).

Plasma lipid analyses: TG and cholesterol determinations. Plasma cholesterol, TG, high density lipoprotein (HDL)-cholesterol, and low density lipoprotein (LDL)-cholesterol were analyzed in a Cobas-Faras centrifugal analyzer using automated enzymatic methods as described by McGowan *et al*. (28) and Allain *et al*. (29).

AT FA analyses. The FA analyses of the AT samples were performed in the laboratory of Dr. Stephen Phinney at University of California at Davis. The procedures have been published previously (19,30).

Statistical analysis. The lipid data and lipoprotein data were analyzed using the paired *T*-test with $n = 10$ or analysis of variance with repeated measures. Analysis of variance was used to determine if Group A and Group B data were comparable. The fatty acid data were analyzed with a single-tailed *T*-test. All the statistical analyses were performed with the PC SAS data system, version 6.11, obtained under license from the SAS Institute, PC-SAS (31).

RESULTS

The addition of 1.5 g/d of AA to a natural food diet for 50 d had no effect on the total plasma lipid levels (See Table 1). The total cholesterol level was identical prior to and after the

TABLE 1
Effect of AA Supplementation on Plasma Cholesterol, Lipoprotein Cholesterol, and TG Concentrations[a]

	Low-AA diet (mg/dL)	High-AA diet (mg/dL)
Total cholesterol	161.90 ± 32.48	161.83 ± 32.23
LDL-cholesterol	89.45 ± 24.72	90.71 ± 20.42
HDL-cholesterol	38.71 ± 7.37	37.79 ± 7.22
TG	95.47 ± 59.75	101.70 ± 61.01

[a]Mean value ± SD, $n = 10$. AA, arachidonic acid; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglyceride.

AA supplementation. As one would expect from the lack of change in the blood lipid levels, the apoprotein levels measured here did not change. Although the Lp(a) did decrease by about 5 mg/dL, this change was not statistically significant either (see Table 2).

Significant changes were detected in the total plasma FA composition in all subjects, with a near doubling of the AA level and 20% reduction in the serum linoleic acid level when they consumed the high-AA diet (see Fig. 1). In Group B there were no significant changes in the FA composition of the subjects tissues during the first 50 d when they consumed the low-AA diet. When the high-AA diet was consumed, a smaller reduction in the plasma oleic acid content was noted, as was a small, but statistically significant, rise in the stearic acid content. None of the other plasma FA levels changed significantly. The changes in the FA composition of the individual lipid classes were also analyzed and showed major differences from the pattern found in the total plasma FA composition. The plasma TG do not contain much AA, about 2% on the low-AA diet. This increased to 3% on the high-AA diet (see Fig. 2). Almost all of the increase was compensated for by a decrease in the oleic acid content of the TG without any significant change in the TG's linoleic acid content. In contrast, the changes in the FA composition of the cholesterol esters (see Fig. 3) were in the AA levels (increased) and the linoleic acid levels (decreased). No other FA showed significant changes in the cholesterol ester fraction. The phospholipid (PL) fraction's FA composition showed the most marked changes after the subjects consumed the AA supplement (see Fig. 4). The AA level rose from 10.3 to 19.0%, and the linoleic acid level dropped from 23 to 17%. There was a slight drop in the level of oleic acid and a slight rise in the level of stearic acid, the source of the increase in stearic acid content

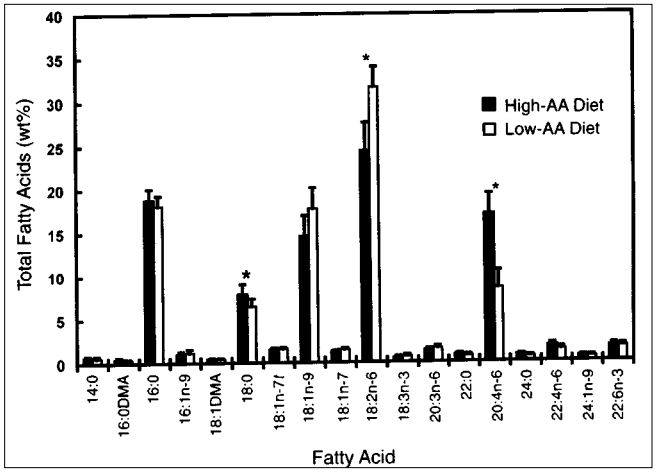

FIG. 1. The mean ($n = 10$) values for the major fatty acids in the subjects' plasma after the subjects had been on the low-arachidonic acid (AA) (65 d) or high-AA diet (50 d). The asterisk indicates fatty acid values that differed significantly by at least $P < 0.05$ between the two dietary periods. The vertical lines define one standard deviation from the means for the 10 subjects in this study. Fatty acids are designated by chain length, number of double bonds in the molecule, and the position of the first double bond from the methylene end of the molecule; DMA, dimethylacetal; *t, trans* fatty acid.

of the total plasma FA noted previously. The plasma free fatty acid (FFA) showed very little overall change except for a small, but statistically significant rise in the AA level in this fraction (see Fig. 5).

AT. The FA composition of the adipose tissue TG is shown in Figure 6, and that of the AT PL in Figure 7. No significant

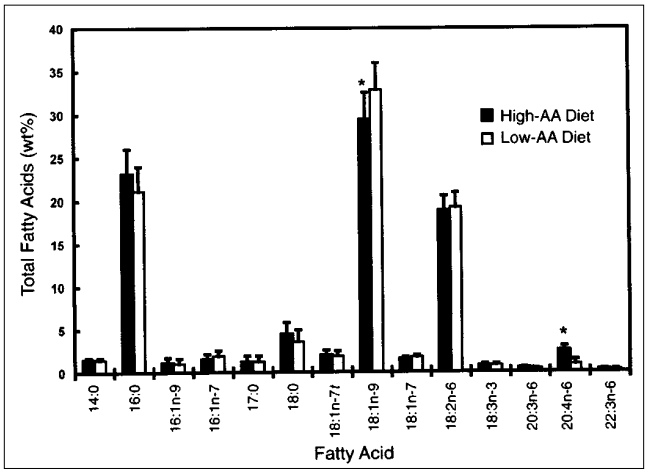

FIG. 2. The mean ($n = 10$) values for the major fatty acids in the subjects' plasma triglyceride fraction after the subjects had been on the low-AA (65 d) or high-AA diet (50 d). The asterisk indicates fatty acid values that differed significantly by at least $P < 0.05$ between the two dietary periods. The vertical lines define one standard deviation from the means for the 10 subjects in this study. Fatty acids are designated by chain length, number of double bonds in the molecule, and the position of the first double bond from the methylene end of the molecule. See Figure 1 for abbreviations.

TABLE 2
Apoprotein (Apo) Levels in Plasma of Subjects After the High-AA or Low-AA diets[a]

	Apoprotein (mg/dL)		
	ApoA-1	ApoB	Lp(a)
High-AA diet	86.75 ± 12.07	69.83 ± 17.58	10.92 ± 18.23
Low-AA diet	87.27 ± 14.71	69.75 ± 17.52	10.15 ± 17.19

[a]Mean value ± SD, $n = 10$. See Table 1 for other abbreviation.

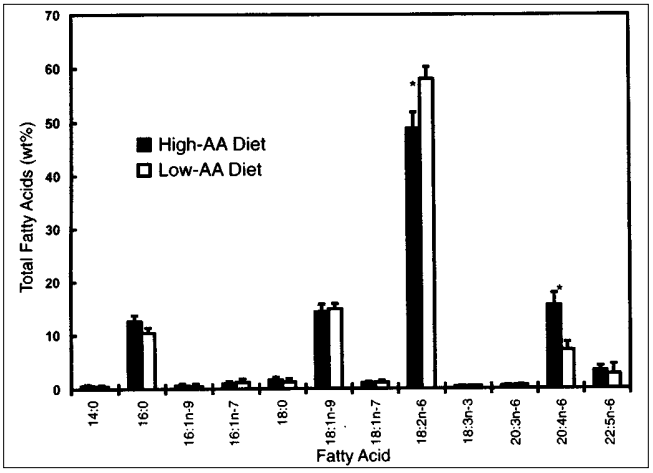

FIG. 3. The mean (n = 10) values for the major fatty acids in the subjects' plasma cholesterol ester fraction after the subjects had been on the low-AA (65 d) or high-AA diet (50 d). The asterisk indicates fatty acid values that differed significantly by at least $P < 0.05$ between the two dietary periods. The vertical lines define one standard deviation from the means for the 10 subjects in this study. Fatty acids are designated by chain length, number of double bonds in the molecule, and the position of the first double bond from the methylene end of the molecule. See Figure 1 for abbreviations.

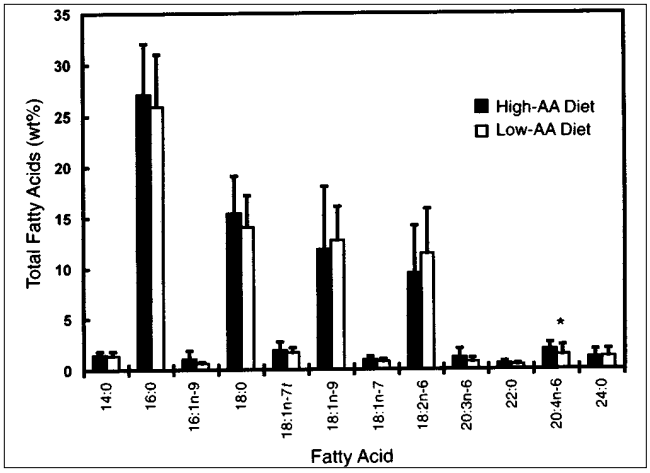

FIG. 5. The mean (n = 10) values for the major fatty acids in the subjects' plasma free fatty acid fraction after the subjects had been on the low-AA (65 d) or high-AA diet (50 d). The asterisk indicates fatty acid values that differed significantly by at least $P < 0.05$ between the two dietary periods. The vertical lines define one standard deviation from the means for the 10 subjects in this study. Fatty acids are designated by chain length, number of double bonds in the molecule, and the position of the first double bond from the methylene end of the molecule. See Figure 1 for abbreviations.

changes in the FA composition were observed after the AA supplementation in either the TG or PL fractions. Table 3 gives the AA and 22:4n-6 levels in these two fractions. There is a trend toward increased AA levels in the PL of the AT after AA supplementation although the trend did not reach statistical significance in either fraction. The TG of AT contained very little AA (<0.5%), and there was no evidence of any increase in the AT TG after the dietary supplementation.

Red blood cells. Figure 8 shows the FA composition of the red cells before and after dietary supplementation with AA. AA supplementation appeared to increase the level of

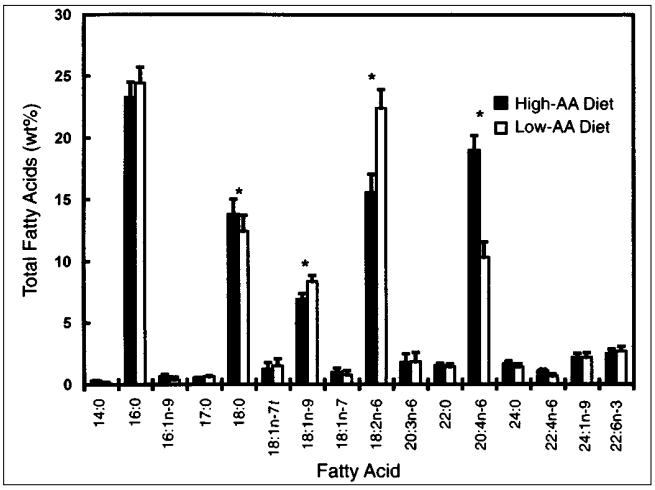

FIG. 4. The mean (n = 10) values for the major fatty acids in the subjects' plasma phospholipid fraction after the subjects had been on the low-AA (65 d) or high-AA diet (50 d). The asterisk indicates fatty acid values that differed significantly by at least $P < 0.05$ between the two dietary periods. The vertical lines define one standard deviation from the means for the 10 subjects in this study. Fatty acids are designated by chain length, number of double bonds in the molecule, and the position of the first double bond from the methylene end of the molecule. See Figure 1 for abbreviations.

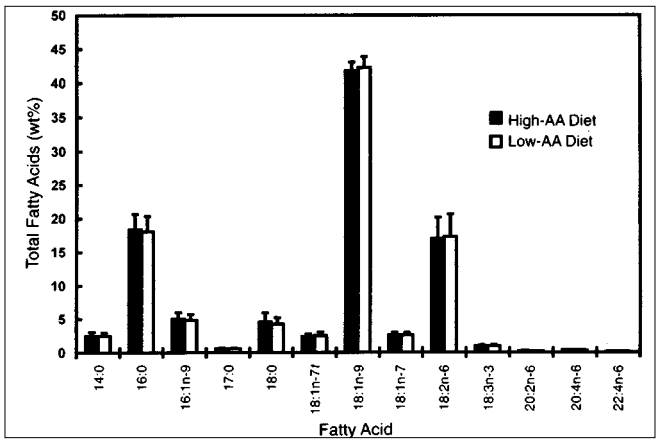

FIG. 6. The mean (n = 10) values for the major fatty acids in the subjects' adipose tissue triglyceride fraction after the subjects had been on the low-AA (65 d) or high-AA diet (50 d). The vertical lines define one standard deviation from the means for the 10 subjects in this study. Fatty acids are designated by chain length, number of double bonds in the molecule, and the position of the first double bond from the methylene end of the molecule. See Figure 1 for abbreviations.

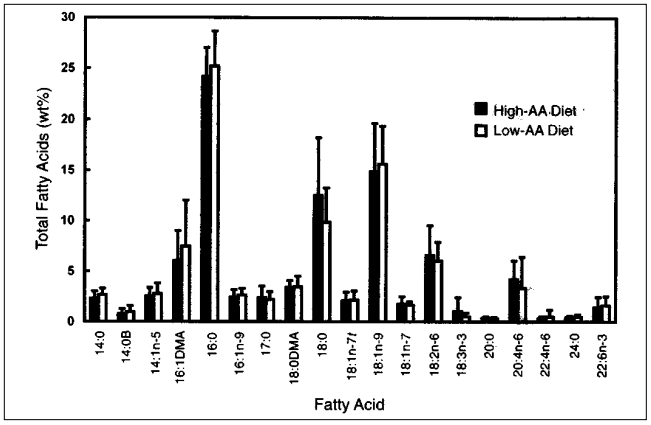

FIG. 7. The mean (n = 10) values for the major fatty acids in the subjects' adipose tissue phospholipid fraction after the subjects had been on the low-AA (65 d) or high-AA diet (50 d). The vertical lines define one standard deviation from the means for the 10 subjects in this study. Fatty acids are designated by chain length, number of double bonds in the molecule, and the position of the first double bond from the methylene end of the molecule. B: branched. See Figure 1 for other abbreviations.

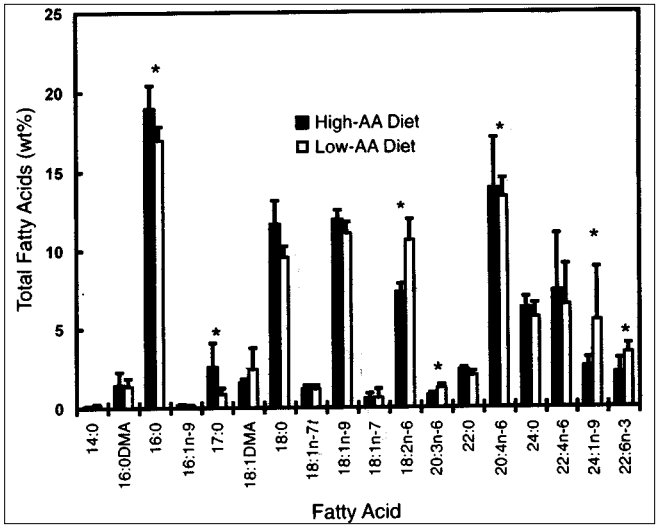

FIG. 8. The mean (n = 10) values for the major fatty acids in the subjects' red blood cells after the subjects had been on the low-AA (65 d) or high-AA diet (50 d). The vertical lines define one standard deviation from the means for the 10 subjects in this study. Fatty acids are designated by chain length, number of double bonds in the molecule, and the position of the first double bond from the methylene end of the molecule. See Figure 1 for abbreviations.

palmitic, stearic, and AA and lower the level of linoleic, nervonic, and docosahexaenoic acid in the red blood cells when AA levels in the diet are increased.

DISCUSSION

When the level of AA in the diet is increased for 50 d from 210 mg/d to 1.7 g/d in an isocaloric substitution for oleic acid, there are no detectable changes in the plasma lipids or lipoprotein profile. The individuals studied in this work were all healthy, normolipidemic subjects (18), and it is more difficult to achieve lipid reductions in normolipidemic persons than in hyperlipidemic subjects (32,33). Also, the polyunsaturated/saturated ratio was held constant in this study at 1:1. Hayes and Khosla (34,35) suggested that plasma lipid levels show little alteration with changes in the amount of FA in the diet if the linoleic acid content of the diet is above 7%, as it was in this study. Lp(a) levels did decrease slightly, but the decrease was not statistically significant, perhaps because the intervention period was too short or the amount of AA fed was too small. Increased levels of dietary n-6 polyunsaturated FA have been reported to decrease HDL and LDL levels in hyperlipidemic subjects (36,37). In this study we added about 0.5% of calories of polyunsaturated n-6 FA while holding the saturated fat content of the diet constant. At this level no effect on the LDL or HDL levels was apparent. Thus, this study suggests that dietary AA at levels found in normal human diets will have no effect on the blood lipid levels or lipoprotein profile in normolipidemic individuals.

In contrast to the blood lipids and lipoproteins, a 1.5 g/d increase in dietary AA has significant effects on the FA profile of the plasma. Normally, the plasma FA profile reflects the dietary FA profile (2,3,38,39). It appears as if dietary AA disproportionately influences the AA level in the plasma. In a typical, fasting normolipidemic individual, there is a pool of 15 g of plasma FA in the circulation. If one calculates the increase in the percentage of AA found in the plasma in this study (from about 8 to 16%), all of one day's dietary AA is found in the plasma. This is in marked contrast to some other FA such as linolenic acid (3) or stearic acid (39) in which very little of the ingested material appears in the plasma FA.

Most of the increase in plasma AA level was found in the plasma PL and cholesterol ester fractions, although the increase in AA content of the plasma cholesterol ester, though significant, was intermediate between the PL and TG levels. The plasma TG showed relatively little increase in their AA levels. One interesting observation was that AA replaced oleic acid in the plasma TG, and primarily linoleic acid in cholesterol esters and PL.

As AA in the plasma FFA is a substrate for cyclooxygenase (40,41), it is probably beneficial that AA level in the

TABLE 3
AA and 22:4n-6 Content of Adipose Tissue Triglycerides and Phospholipids After High-AA or Low-AA Diets[a]

	Lipid (mean value ± SD, n = 10)		
Lipid fraction	Fatty acid	High-AA diet	Low-AA diet
Triglyceride	20:4n-6	0.33 ± 0.07	0.30 ± 0.07
	22:4n-6	0.11 ± 0.02	0.10 ± 0.02
Phospholipid	20:4n-6	4.22 ± 1.85	3.31 ± 3.14
	22:4n-6	0.33 ± 0.16	0.48 ± 0.68

[a]wt% of total fatty acids. See Table 1 for abbreviation.

plasma FFA is only marginally influenced by dietary AA. Here we found that AA level in the FFA in plasma was 2% in subjects on the low-AA diet and increased to 3% when they consumed to high-AA diet. The analytical variation in the level of other FA present in the plasma FFA was large, but none showed any statistically significant variation before and after consumption of the high-AA diet in these subjects.

The lack of deposition of AA in the AT was interesting, particularly in view of the data of Phinney *et al*. (19,30), suggesting that weight loss alone can have a significant effect on the AA content of the AT. In human tissues, AA is not present in high levels in TG (2,30,42). Here we found only a small increase in the AA level in the plasma TG and no increase in the level in AA in the AT. These subjects were in caloric balance. Weight loss or gain was restricted to less than 2% of their body weight at entry into the study (16). Apparently, none of the 75 g of AA consumed during the 50 d of the intervention was stored in the AT of these subject during that period except as a direct exchange with AA already present in the tissue. The reason for this is not known, but we can speculate that (i) the increased dietary AA is rapidly metabolized to other products and, hence not available for incorporation into AT, or (ii) that the turnover of FA in the AT is so slow in subjects with constant weight that an increase in the AA level could not be detected under the conditions of this experiment.

The half-life of red blood cells (RBC) is approximately 120 d in humans. Thus, the RBC FA composition data presented here represent a nonequilibrium state and, hence, are difficult to interpret. It is interesting, however, to note that the increase in the AA level was small, but the decrease in the linoleic acid level in the red cells was much larger. The decrease in linoleic acid was compensated for by increases in the palmitic and stearic acid levels rather than the AA level in the RBC of these subjects. There are no readily apparent explanations for these changes. One can speculate, however, that increased dietary AA interferes with incorporation of linoleic acid into RBC. The RBC then compensate by incorporating *in vivo* synthesized palmitic and stearic acid into their PL in place of linoleic acid.

In conclusion, this study demonstrates that dietary AA in a level eightfold above that found in a typical Western diet has little or no effect on blood lipid, lipoprotein, or apoprotein levels. Dietary AA at these levels does markedly alter the AA levels in the plasma, particularly the plasma PL. Also, dietary AA is not readily incorporated into AT. The effects of dietary AA on RBC FA are complex and not readily interpreted in a 50-d study. In terms of blood lipids and lipoproteins that are risk factors for cardiovascular disease, there are no obvious benefits or adverse effects from consuming an eightfold increase in dietary AA for 50 d.

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Increased Dietary Arachidonic Acid Enhances the Synthesis of Vasoactive Eicosanoids in Humans

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ABSTRACT: Data on the effect of dietary arachidonic acid (AA) (20:4n-6) on the synthesis of thromboxane and prostacyclin (PGI_2) in humans are lacking. We measured the effect of 1.5 g/d (*ca.* 0.5 en%) of 20:4n-6 added isocalorically to a stabilization (low-AA) diet on the excretion of 11-dehydrothromboxane B_2 (11-$DTXB_2$) and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ (PGI_2-M). In a crossover design, 10 healthy men, living in a metabolic unit, were fed a diet (low-AA) containing 210 mg/d of 20:4n-6 for 65 d and an identical diet (high-AA) that contained 1.5 g/d of additional 20:4n-6 for 50 d. Three-day urine pools were collected at the end of each dietary period and analyzed for eicosanoids by gas chromatography–electron capture negative ion–tandem mass spectrometry. Mean excretion of 11-dehydrothromboxane B_2 was 515 ± 76, 493 ± 154, and 696 ± 144 ng/d (SD; $n = 10$) during the acclimation (15 d) low-AA diet and high-AA diet periods, respectively (41% increase from low-AA to high-AA diet, $P = 0.0037$); mean excretion of PGI_2-M was 125 ± 40, 151 ± 36, and 192 ± 55 ng/d (SD; $n = 10$) during acclimation (15 d) low-AA and high-AA diets, respectively (27% increase from low-AA to high-AA diets; $P = 0.0143$). Thus, both the metabolites of thromboxane and PGI_2 increase on the high-AA diet. Furthermore, both indicated changes in metabolite excretion may be associated with measurable effects on several physiologically significant cellular functions, such as platelet aggregation *in vivo* and inflammation in response to immune challenges.

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Eicosanoids are a group of oxygenated metabolites of arachidonic acid (AA) (20:4n-6) which are ubiquitous in mammalian tissues. They produce a remarkably broad spectrum of effects that embraces nearly every biological function. Hence, inhibition and enhancement of their biosynthesis can be important tools of therapeutic strategies. In 1971 Vane (1) demonstrated that aspirin inhibits the synthesis of eicosanoids, specifically those arising through the cyclooxygenase pathway, and that this inhibition is the mechanism of action of aspirin-like drugs. Many other drugs have since been shown to alter eicosanoid production.

The immediate biochemical precursor of all the major physiologically active eicosanoids is AA which is normally present in very small quantities in the human diet (approximately 200 mg/d) but can be synthesized, in the human, from linoleic acid (18:2n-6), a much more abundant lipid component in the occidental diet (2). This relationship justifies the hypothesis that the endogenous synthesis of eicosanoids can also be influenced by manipulations of dietary polyunsaturates. Indeed, it has been shown that prostaglandin E_2 synthesis can be increased by supplementing the intake of AA (3). Nonprostaglandin precursor fatty acids, e.g., saturated fatty acids, have also been suggested to modulate eicosanoid biosynthesis (4,5).

Thromboxane (TXA_2), a powerful inducer of platelet aggregation and vasoconstriction, and prostacyclin (PGI_2), one of the most potent hypotensive and antiaggregatory agents, are key eicosanoids in vascular physiology. According to a model developed by Moncada and Vane (6), PGI_2 and TXA_2 represent biologically opposite poles of a mechanism for regulating platelet-vessel wall interaction and the formation of the hemostatic plug and intra-arterial blood clots. A number of groups have found that dietary eicosapentaenoic (20:5n-3) acid, a major component of marine lipids, tends to reduce arachidonate metabolism to thromboxane and PGI_2 in humans (7–13). Studies in humans (12,13), monkeys (14), and rats (15), however, have also been reported to show that dietary fish oil does not reduce the rate of PGI_2 synthesis. Given the broad spectrum of biological activities of eicosanoids, it is conceivable that an increase in their output may be desirable under certain clinical and/or experimental circumstances. Whelan *et al.* (16,17) have recently demonstrated that AA is a more effective eicosanoid modulator in mice than is eicosapentaenoic acid, both *in vitro* and *in vivo*. In humans, there are no reported data on the effect of dietary 20:4n-6 on the endogenous output of TXA_2 and PGI_2. Additionally, it is of interest to learn what effect, if any, AA will have on eicosanoid output in diets already rich in linoleic acid. In the

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Abbreviations: 11-$DTXB_2$, 11-dehydrothromboxane B_2; GC–ECNI–MS–MS, gas chromatography–electron capture negative ion–tandem mass spectrometry; PGI_2, prostacyclin; PGI_2-M, 2,3-dinor-6-oxo-prostaglandin $F_{1\alpha}$; TXA_2, thromboxane A_2.

present study conducted with 10 volunteers, we quantified the effect of an isocaloric addition of AA (*ca.* 0.5 energy percentage, or en%) on the excretion of 11-dehydrothromboxane B_2 (11-DTXB_2) and 2,3-dinor-6-oxo-PGF$_{1\alpha}$ (PGI_2-M).

MATERIALS AND METHODS

Subjects. Initially, 12 healthy, nonobese male subjects between the ages of 20 and 38 were selected from residents of the San Francisco Bay area. Those who met preliminary selection criteria (nonsmoking, relatively stable body weight, no history of alcohol abuse, and moderate physical activity) were given a physical examination and were subjected to hematological and chemical screening. Those with test results within normal ranges were entered into the study. They were housed at the Human Nutrition Suite, Western Human Nutrition Research Center, USDA, ARS, Presidio of San Francisco, California, for the duration of the study (130 d). They were required not to ingest aspirin or any other anti-inflammatory agents and to report any medication prescribed by a physician during the study. The subjects were fully informed of the purpose of the study, procedures to be followed, samples to be collected, risks, benefits, and payment associated with the study. All procedures were approved by the University of California, Davis, Human Subjects Committee, and the USDA, ARS Human Studies Review Committee.

Controlled diets. Two low-fat diets, designated as low-AA (stabilization) and high-AA (intervention) diets, consisted of natural foods purchased from local food suppliers. All nutrients, including micronutrients, were provided by the diets in amounts to meet or exceed the Recommended Daily Allowances (RDA) (18) as estimated from data in *USDA Handbook 8* (19). The amount of vitamin E was estimated to be twice the RDA. Such excess was intended to counteract any increase in oxidative stress induced by the increase in polyunsaturated fatty acid intake in the high-AA diet. The high-AA diet was identical to the low-AA diet except that it contained 3 g of a triglyceride (called ARASCO®) produced by the Martek Biosciences Corporation (Columbia, MD) from algae. ARASCO® oil, which was 50% AA, isocalorically replaced the high-oleic safflower oil used in the stabilization (low-AA) diet. The study used a 5-d rotating menu cycle, and no food or food supplement other than what was provided by the study was allowed. *Handbook 8*, *The Composition of Foods*, (19) and in-house nutrient databases were utilized to determine the nutrient composition of the diets.

Duplicate sets of 5-d representative composites of both diets were analyzed separately for fatty acids by gas–liquid chromatography. Table 1 shows the mean fatty acid composition in the two diets. Energy contribution (en%) from proteins, carbohydrates, and lipids was virtually identical in both diets: 15%, 55%, and 30%, respectively. Alcoholic beverages were not allowed, whereas consumption of decaffeinated coffee or tea, and water was unrestricted. The energy values of the meals were adjusted, daily if necessary, to maintain the participants' body weights. We made adjustments so that the fatty acid composition of the diet remained constant. The calories were reduced (or increased) by reducing (or increasing) the subject's intake of all nutrients proportionally, while the 1.5 g/d of AA remained constant. Thus, the average weights of the subjects did not vary significantly during the 130 d of the study. The mean energy intake for all subjects during the entire study was about 2800 Kcal/d. Further details concerning the diets have been reported elsewhere (20).

TABLE 1
Fatty Acids (%) in the Two Diets[a]

Fatty acid	Low-AA diet	High-AA diet
12:0	0.61	0.72
14:0	2.38	2.45
14:1n-5	n.d.[b]	0.16
15:0	0.29	0.30
16:0	15.34	15.25
16:1n-9	0.83	0.81
17:0	0.31	0.31
18:1n-9DMA[c]	0.24	0.21
18:0	7.67	7.66
18:1n-7*trans*	6.29	6.11
18:1n-9	34.22	31.32
18:1n-7	1.53	1.46
18:1n-5	1.48	1.47
18:2*trans-trans*	0.51	0.56
18:2n-6	23.80	23.91
18:3n-6	0.37	0.39
18:3n-3	1.86	1.94
20:1n-11	0.25	0.30
22:0	0.26	0.29
20:4n-6	0.23	2.16
Total	98.47	97.78
Unknowns	1.53	2.22

[a]Average values from the analysis of 10 diet composites.
[b]n.d., None detected.
[c]Dimethyl acetal of (9*Z*)-octadecenal.

Experimental protocol. All subjects were initially fed diet low-AA containing 210 mg/d of AA from natural foods. On day 15, one-half of the subjects (Group A) switched to a diet (high-AA) identical to the low-AA diet except that it contained 3 g of a triglyceride consisting of 50% 20:4n-6, while the others (Group B) remained on the low-AA diet. On day 65, the two groups were crossed over: Group B now received the high-AA diet and Group A received the low-AA diet. On day 115, Group A was returned to the low-AA diet. All subjects were released on day 130.

Urine collection. For eicosanoid analysis, 24-h urine samples were collected in polyethylene bottles and kept refrigerated during the collection period. Urine was collected during days 13 to 15, 63 to 65, 113 to 115, and 128 to 130. After the 24-h collections were completed, urine volumes were measured, and 2% portions of each 24-h (three consecutive day) collection were pooled and stored at −70°C until analyses could be performed. The subjects, who had no access to normal toilet facilities, were under constant supervision and received detailed instructions about urine collection.

Measurement of 11-DTXB_2 and PGI_2-M. Analyses of 11-DTXB_2 and 2,3-dinor-6-oxo-PGF$_{1\alpha}$ (PGI_2-M) were per-

formed on 10-mL aliquots of the 72-h pools prepared as described above. This enabled us to assess the mean total synthesis of TXA_2 and PGI_2 during the 72-h periods. Specifically, to estimate the effect of dietary changes on eicosanoid excretion, analyses were performed on days 63–65 (high-AA diet) and days 128–130 (low-AA diet) urine pools in Group A subjects. In Group B subjects, the analyses were performed on days 63–65 (low-AA diet) and days 113–115 (high-AA diet) urine pools. (See scheme of study depicted in Ref. 20). Quantification was achieved by capillary gas chromatography–electron capture negative ion–tandem mass spectrometry. As reported previously, the interassay coefficient of variation (precision) for the 11-$DTXB_2$ assay was 1–2% (21) and about 5% for PGI_2-M (22). Detailed procedures and instrumental conditions were described previously (21,22).

Statistical analysis. We evaluated the 11-$DTXB_2$ and PGI_2-M 24-h excretion rates and AA intakes using the PROC MIXED package of the SAS Institute (Cary, NC). *P* values less than 0.05 were considered statistically significant.

RESULTS

Two subjects, both from Group B, withdrew from the study. We were thus left with a 6:4 subject distribution between the groups. The 11-$DTXB_2$ and PGI_2-M data were tested for carryover effects, and none achieved statistical significance ($F = 1.87$, $P = 0.2083$; $F = 2.27$, $P = 0.1705$, respectively). We also compared the fatty acid composition of plasma, platelets, and red cells on day 15 to day 130 (23,24). As they were identical, we assumed that the washout period was adequate in this regard. The mean 11-$DTXB_2$ urinary excretion was 515 ± 24 ng/d after 15 d of the low-AA diet, 493 ± 49 ng/d (SE; $n = 10$) after 65 d of the low-AA diet, and 696 ± 45 ng/d with the high-AA diet (41% increase, $P = 0.0037$); the mean PGI_2-M excretion at these same points was 125 ± 13 ng/d, 151 ± 11 ng/d and 192 ± 17 ng/d (SE; $n = 10$), respectively (27% increase, $P = 0.0143$). Excretion values for individual subjects are shown in Tables 2 and 3. Values at the end of the two diet periods for each of the 10 individuals who completed the study are shown graphically in Figures 1 and 2.

DISCUSSION

This study quantifies the effect of an eightfold increase in AA intake on the biosynthesis of TXA_2 and PGI_2 as measured by the urinary excretion of their respective metabolites. Ten male volunteers, when subjected to isocaloric supplementation with 1.5 g/d of AA, synthesized an average of 41% more TXA_2 and 27% more PGI_2 than they did when they were consuming a stabilization diet containing 210 mg/d of AA. The magnitude of these increases is, in our opinion, remarkable as they occurred through the action of only 0.5 en% of added AA and in the presence of relatively large quantities of dietary linoleate, the precursor of AA (Table 1). In contrast, a previous study supplementing dietary AA at a fourfold higher level than in our study found a rather modest increase in PGE metabolite excretion (3). Under normal physiological conditions, platelets provide approximately 80% of TXA_2. Whereas endothelial cells are the major source of PGI_2, there is evidence that in rats the gastrointestinal tract is another site of PGI_2 synthesis from dietary precursor fatty acids (25). However, to our knowledge, it has not been demonstrated that in humans PGI_2 is absorbed through the gut and thereby subjected to the normal catabolic pathway leading to PGI_2-M. An increase in PGI_2 production in response to AA supplementation has been previously observed in rats, both *in vitro* and *in vivo*, by Mann *et al.* (26). The authors also observed that there was a significant positive correlation between the two methods: *in vitro* production of 6-oxo-$PGF_{1\alpha}$ by arterial preparations and *in vivo* PGI_2 production as measured by the urinary level of PGI_2-M.

Our study confirms previous observations (12–14) that the synthesis of TXA_2 is more accessible than the synthesis of PGI_2 to diet-induced alterations. Indeed, in a fish-oil supple-

TABLE 2
Mean Urinary Excretion Rates (ng/24 h) of 11-Dehydrothromboxane B_2 During the Last Three Days of Each Dietary Treatment

Subject[a]	Day 15	Diet		Increase[b] (%)
		Low-AA	High-AA	
1	449 (435–462)[c]	374 (371–377)[c]	550 (536–564)[c]	47.1
2	539 (528–550)	658 (654–661)	691 (676–705)	5.0
3	416 (408–424)	556 (549–564)	720 (709–731)	29.5
5	397 (396–398)	325 (322–327)	509 (492–526)	56.6
7	625 (606–643)	631 (630–631)	768 (752–785)	21.7
8	537 (520–553)	386 (378–394)	645 (640–650)	67.6
9	582 (562–602)	404 (388–420)	866 (852–880)	114.4
10	538 (525–550)	647 (647–648)	974 (966–981)	50.5
11	486 (480–492)	277 (269–285)	646 (612–680)	133.2
12	581 (567–595)	669 (658–680)	594 (582–606)	–11.2
Mean ± SD	515 ± 76	493 ± 154	696 ± 144[d]	

[a]Subjects 4 and 6 withdrew from the study.
[b]Percentage increase calculated as [(high-AA minus low-AA)/low-AA] × 100.
[c]Range in parentheses, $n = 2$.
[d]Low-AA compared with high-AA diet; $P < 0.004$.

TABLE 3
Mean Urinary Excretion Rates (ng/24 h) of 2,3-Dinor-6-oxo-prostaglandin F1α During the Last Three Days of Each Dietary Treatment

Subject[a]	Day 15	Diet		Increase[b] (%)
		Low-AA	High-AA	
1	134 (133–134)[c]	173 (172–174)[c]	235 (224–247)[c]	35.8
2	115 (113–117)	170 (161–179)	164 (164–165)	–3.5
3	100 (99–101)	124 (121–127)	195 (192–198)	57.3
5	136 (131–140)	193 (184–203)	206 (204–207)	6.7
7	111 (110–112)	150 (150–150)	171 (164–179)	14.0
8	131 (131–132)	126 (122–130)	183 (181–185)	45.2
9	229 (229–230)	208 (182–234)	323 (297–348)	55.3
10	83 (82–84)	126 (126–127)	153 (151–156)	21.4
11	119 (118–120)	89 (89–89)	170 (155–186)	91.0
12	99 (99–100)	148 (144–152)	121 (119–123)	–18.2
Mean ± SD	125 ± 40	151 ± 36	192 ± 55[c]	

[a]Subjects 4 and 6 withdrew from the study.
[b]Percentage increase calculated as [(high-AA minus low-AA)/low-AA] × 100.
[c]Range in parentheses, $n = 2$.
[d]Low-AA compared with high-AA diet; $P < 0.014$.

mentation (15 g/d) study conducted at the USDA Center (Beltsville, MD), the mean 11-DTXB$_2$ excretion was reduced by 38% while the mean excretion of PGI$_2$-M was reduced by only 22% after 10 wk, compared to a basal diet (9,10). A similar observation was made by Sanigorski *et al.* (27) in a rat study whose results demonstrated the ability of n-3 fatty acids, in this case provided as 18:3 n-3 from linseed oil, to selectively decrease TXA$_2$ production while having little or no effect on PGI$_2$-M production *in vitro*. The greater TXA$_2$ response may be related to the magnitude of diet-induced changes in arachidonate and eicosapentaenoate in platelet and endothelial lipid stores. Because of the antagonistic physiological activities of TXA$_2$ and PGI$_2$-M (as regards platelet aggregation and vasoconstriction), the greater TXA$_2$ response observed in this study (41% vs. 27% increase) might become significant, in terms of increased risk of thrombosis, with increasing dietary AA intake. The effects of the high-AA diet on plasma, platelet and tissue fatty acid composition, platelet function and blood clotting, plasma lipoprotein distribution and apoproteins from this study are reported elsewhere (23,24). Although changes in platelet function and bleeding time were not detected in our healthy, nonsmoking subjects, this may not be true in patients with vascular disease.

The results of this study, together with those of our fish oil study (9,10), agree with observations made by Whelan *et al.* (16,17) in studies with mice and hamsters that AA is a more effective modulator of eicosanoid biosynthesis than n-3 marine fatty acids. Similarly, Sanigorski *et al.* (28) demonstrated that in rats fed high-fat diets, 30 mg/d of dietary AA abolishes the depression of TXA$_2$ and PGI$_2$ production induced by 100 mg/d of n-3 polyunsaturated fatty acids. As shown in Table 1, and as previously stated in the Materials and Methods section under *Controlled diets*, the higher 20:4n-6 intake in the high-AA diet is largely at the expense of oleic acid. To our knowledge, there is no support in the literature for the proposition that the observed shift of the eicosanoid balance is attributable to the minor reduction of oleic acid intake. Thus, in this study we are possibly observing a pure AA effect. Finally, it

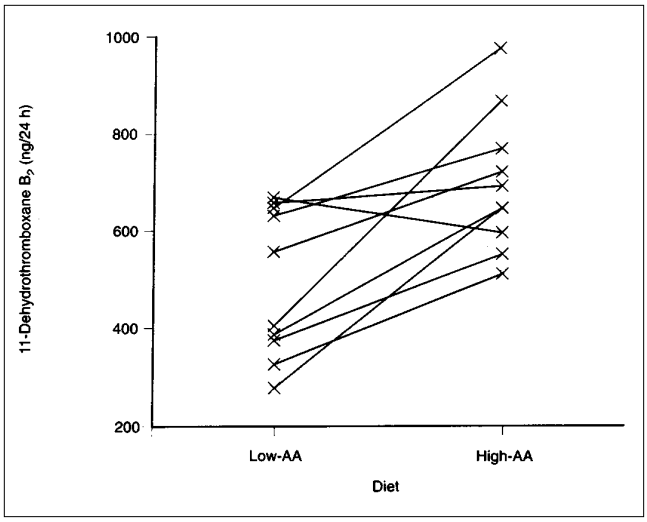

FIG. 1. 11-Dehydrothromboxane B$_2$ excretion rates of the 10 subjects who completed the study; AA, arachidonic acid.

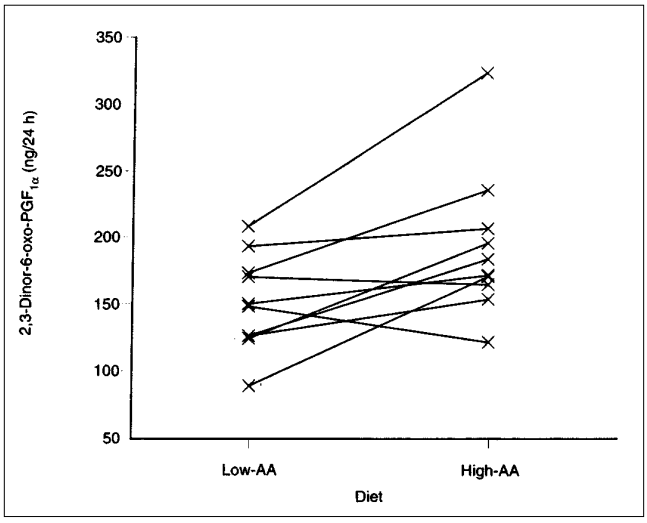

FIG. 2. 2,3-Dinor-6-oxo-PGF$_{1\alpha}$ excretion rates of the 10 subjects who completed the study. See Figure 1 for abbreviation.

is noteworthy that, with regard to both 11-DTXB$_2$ and PGI$_2$-M excretion rates, subject 12 exhibited a response to the high-AA diet which is contrary to the response of all the other subjects. Available data do not allow us to conclude whether this is an example of biological variability or the consequence of unknown factors.

ACKNOWLEDGMENTS

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Influence of Dietary Arachidonic Acid on Metabolism *in vivo* of 8*cis*,11*cis*,14-Eicosatrienoic Acid in Humans

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ABSTRACT: This study investigated the influence of dietary arachidonic acid (20:4n-6) on Δ5 desaturation and incorporation of deuterium-labeled 8*cis*,11*cis*,14-eicosatrienoic acid (20:3n-6) into human plasma lipids. Adult male subjects ($n = 4$) were fed diets containing either 1.7 g/d (HI20:4 diet) or 0.21 g/d (LO20:4 diet) of arachidonic acid for 50 d and then dosed with a mixture containing ethyl esters of 20:3n-6[d4] and 18:1n-9[d2]. A series of blood samples was sequentially drawn over a 72-h period, and methyl esters of plasma total lipid, triacylglycerol, phospholipids, and cholesteryl ester were analyzed by gas chromatography–mass spectrometry. Based on the concentration of 20:3n-6[d4] in total plasma lipid, the estimated conversion of 20:3n-6[d4] to 20:4n-6[d4] was 17.7 ± 0.79% (HI20:4 diet) and 2.13 ± 1.44% (LO20:4 diet). The concentrations of 20:4n-6[d4] in total plasma lipids from subjects fed the HI20:4 and LO20:4 diets were 2.10 ± 0.6 and 0.29 ± 0.2 μmole/mL plasma/mmole of 20:3n-6[d4] fed/kg of body weight. These data indicate that conversion of 20:3n-6[d4] to 20:4n-6[d4] was stimulated 7–8-fold by the HI20:4 diet. Phospholipid acyltransferase was 2.5-fold more selective for 20:3n-6[d4] than 18:1n-9[d2], and lecithin:cholesterol acyltransferase was 2-fold more selective for 18:1n-9[d2] than 20:3n-6[d4]. These differences in selectivity were not significantly influenced by diet. Absorption of ethyl 20:3n-6[d4] was about 33% less than ethyl 18:1n-9[d2]. The sum of the n-6 retroconversion products from 20:3n-6[d4] in total plasma lipids was about 2% of the total deuterated fatty acids. Neither absorption nor retroconversion appears to be influenced by diet.

Lipids 32, 441–448 (1997).

The ratio of 20:3n-6 to 20:4n-6 in tissues is involved in the regulation of the balance between the series-1 and series-2 eicosanoids which effects their physiological impact. Because dihomo-γ-linolenic acid (20:3n-6) and arachidonic acid (20:4n-6) are substrates for the synthesis of these eicosanoids, the possibility has been suggested that diets rich in 20:4n-6 decrease the ratio of 20:3n-6 to 20:4n-6 in tissues and may have a negative effect on a variety of chronic diseases such as heart disease, arthritis, asthma, and hypertension (1–3). Conversely, supplementation of diets with the precursor of 20:3n-6, γ-linolenic acid (18:3n-6), has been reported to have beneficial health effects (1–4). For these reasons, a better understanding of the influence of dietary 20:4n-6 on 20:3n-6 metabolism has potential nutritional and health importance.

A number of studies have used isotope-labeled 18:3n-6 and 20:3n-6 to investigate the metabolism of 20:3n-6 in animal models and *in vitro* (1,5). The metabolism of 20:3n-6 in human subjects has been investigated in studies from two groups (6,7), and there is no information available for the effect of dietary 20:4n-6 on 20:3n-6 metabolism in man. Of the two human studies that have investigated the metabolism of 20:3n-6, one study investigated the effect of single and multiple oral doses of unlabeled 20:3n-6 on the ratio of 20:3n-6 to 20:4n-6 in plasma, platelet, and red cell lipids of three volunteers. The other study used deuterium-labeled 20:3n-6 to follow incorporation and desaturation in four normal and four diabetic subjects. In neither study was the fatty acid composition of the subjects' diets controlled. Results from both studies provide evidence that in humans the conversion of 20:3n-6 to 20:4n-6 by Δ5 desaturation is low In contrast, Δ5-desaturase activity in rats and mice is much higher than in humans (5,6).

This metabolism study in adult male subjects was part of a dietary arachidonic acid study that investigated the effect of dietary 20:4n-6 on platelet function, eicosanoid production, lipoprotein concentrations, immune status, and the fatty acid composition of plasma, red cell, and adipose lipids. The questions addressed by this report are whether the addition of 20:4n-6 to a human diet alters the desaturation of 20:3n-6 to 20:4n-6, the absorption and incorporation of 20:3n-6 into plasma lipid classes relative to 18:1n-9, or the retroconversion of 20:3n-6 to 18:3n-6 and 18:2n-6.

EXPERIMENTAL PROCEDURES

Deuterated fatty acids. Deuterium-labeled oleic acid (9*cis*-18:1-9,10-d2) and dihomo-γ-linolenic acid (8,11,14-20:3-17,17,18,18-d4) were synthesized, purified, and converted to their ethyl esters by previously described methods (8-10). The isotopic purity for 9*cis*-18:1-9,10-d2 was 79.5% d2, 18.4%

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Abbreviations: CE, cholesteryl ester; GC–MS, gas chromatography–mass spectrometry; PL, phospholipid; TG, triglyceride; TLC, thin-layer chromatography; TL, total lipid.

TABLE 1
Fatty Acid Composition of Diets[a]

Fatty acid	LO20:4 (%)	HI20:4 (%)	LO20:4 (g/d)	HI20:4 (g/d)	Difference (g/d)
Saturates	25.8	25.9	21.8	22.4	–0.65
t-18:1	6.4	6.3	5.4	5.4	+0.05
c-18:1	36.9	33.7	31.1	29.1	+1.96
18:2n-6	23.8	23.8	20.1	20.6	–0.52
18:3n-3	1.8	1.9	1.5	1.6	–0.13
20:4n-6	0.2	2.2	0.24	1.7	–1.53
Other	3.6	3.9	3	3.4	–0.34
Unknown	1.5	2.3	1.3	2	–0.72
Total	100	100	84.3	86.5	+2.2

[a]Diets: 2800 total calories; 27.1–27.8 energy percentage (en%) total fat, 15 en% protein, 57 en% carbohydrate.

d1, 2.0% d0 and 93.4% d4, 3.6% d3, 2.9% d5 for 8,11,14-20:3-17,17,18,18-d4. Chemical purity was 98.8% for 9c-18:1-9,10-d2 and 98.3% for 8,11,14-20:3-17,17,18,18-d4. The impurities in these samples were *trans* isomers, and the 20:3-17,17,18,18-d4 sample contained no deuterium-labeled 20:4n-6.

Study design. The subjects were four Caucasian males between the ages of 20 and 28. Medical histories, physical examinations, and clinical blood profile data indicated that the subjects were in good health and had no history of congenital ailments. The subjects' height/weight ratios, blood pressure, fasting serum cholesterol and triglyceride (TG) concentrations were within normal ranges. Serum TG values were 217 and 143 mg/dL, and total cholesterol values were 190 and 139 mg/dL for the subjects in the LO20:4 diet group. Serum TG concentrations were 37 and 64 mg/dL, and total cholesterol was 133 and 146 mg/dL for subjects in the HI20:4 diet group. Institutional ethical approval was obtained for the study protocol from the Agricultural Research Service's Human Studies Review Committee (Washington, D.C.). Informed consent was obtained from each subject prior to initiation of the study.

The subjects were housed in a metabolic ward and provided diets that contained 1.7 g of 20:4n-6 (HI20:4) or 0.21 g of 20:4n-6 (LO20:4). ARASCO® oil (3 g/d), provided by Martek Bioscience Corp. (Columbia, MD), was added as the source of 20:4n-6 in the HI20:4 diet. An equal amount of high-oleic safflower oil (3 g/d) was added to the LO20:4 diet. The subjects were fed these diets for 50 d prior to being fed the mixture of deuterated fatty ethyl esters. The fatty acid composition of the diets is summarized in Table 1. The ARASCO® oil contained 0.025% of tocopherol and 0.025% of ascorbyl palmitate as antioxidants. The peroxide value was 0.4 meq/kg. Analysis by gas chromatography (GC), GC–mass spectrometry (MS), and thin-layer chromatography (TLC) detected only traces of oxidation products. The fatty acid composition of the ARASCO® oil (wt%) was: 0.5% 14:0; 0.2% 15:0; 11.1% 16:0; 0.5% 17:0; 10.6% 18:0; 8.7% 9*c*-18:1; 0.2% 11*c*-18:1; 6.5% 18:2n-6; 3.8% 18:3n-6; 0.9% 20:0; 0.4% 20:1n-9; 0.6% 20:2n-6; 0.6% 5,11,14-20:3; 2.5% 20:3n-6; 49.7% 20:4n-6; 1.6% 22:0; 1.2% 24:0; 0.2% 22:4n-6. Trace amounts of other fatty acids are not listed. Complete detailed information for the diet compositions is described elsewhere (11).

All meals were prepared from weighed food portions and their consumption monitored. Duplicate meals were collected and analyzed for fatty acid composition. Values for total fat, protein, and carbohydrate were obtained by analysis of diet composites. The data used by this computer program are based mainly on the food composition data in a USDA handbook (12). No significant changes in the subjects' weights were observed during the controlled diet period, indicating a stable energy balance. The subjects fasted for 12 h before the experimental meals were fed.

The deuterated 18:1n-9[d2] and 20:3n-6[d4] ethyl esters were mixed with 112 g of no-fat yogurt, and the mixtures were fed between 7:00 and 7:30 a.m. Subjects were provided a no-fat breakfast at 8:00 a.m. to avoid dilution of the labeled fatty acid with exogenous fatty acids. A low-fat (*ca*. 15% fat calories) lunch was provided at 12:00, and the usual diets were fed at 5:00 p.m. and on subsequent days. The amount of deuterated fatty acid ethyl esters in the mixture fed to each subject is given in Table 2. The μmole data for the deuterated fatty ethyl esters fed have been adjusted by multiplying the weighted amounts by their percent isotopic and chemical purity.

Sample collection. Blood samples (*ca*. 14 mL each) were collected by venipuncture at 0, 4, 6, 8, 12, 24, 48, and 72 h for analysis of plasma lipid class fatty acids. Additional blood samples (*ca*. 11 mL) were collected at 2, 4, 6, and 8 h for analysis of the chylomicron total lipid. Standard preparative ultracentrifuge methods (13) were used to separate the chylomicron fraction. An aliquot of the chylomicron samples was analyzed by electrophoresis to confirm the purity of the chylomicron fractions (14).

TABLE 2
Deuterium-Labeled Fatty Esters in Mixtures Fed

Subject code	Body weight (kg)	μmole ^{2}H fat/kg body weight[a]		18:1n-9[d_2] (%)	20:3n-6[d_4] (%)
		18:1n-9[d_2]	20:3n-6[d_4]		
HG3	67.6	76.5	58.9	56.5	43.5
HG9	73.6	66.7	59.8	52.7	47.3
HG8	73.6	68.4	55.6	55.2	44.8
HG10	85.0	61.4	39.9	60.6	39.4

[a]Data include corrections for isotopic and chemical purity.

Analysis of plasma lipid fatty acids. Plasma total lipids (TL) were extracted with 2:1 chloroform/methanol (15). Preparative TLC was used to isolate TG, cholesteryl ester (CE), and phospholipid (PL) from plasma lipids (16,17). Known weights of triheptadecanoin, cholesteryl heptadecanoate, and diheptadecanyl-*sn*-phosphatidylcholine (Applied Science, State College, PA) were added as internal standards to the TL extract. The 17:0 internal standard data were used to determine the concentrations (μmole/mL) of each deuterated and nondeuterated fatty acid in the plasma lipid classes. Methyl esters of the isolated lipid classes were prepared by heating the samples with a 5% HCl–methanol solution (10). Quantitative analysis of the deuterium-labeled fatty acids incorporated into plasma and lipoprotein lipids was by GC–MS analysis of their methyl esters (18).

Fatty acid percentages and concentrations for both labeled and unlabeled fatty acids were obtained by analysis of their methyl esters with a Hewlett-Packard model 5988A quadrupole mass spectrometer (Palo Alto, CA) operated in a positive chemical ionization mode with isobutane as the ionization reagent. The GC–mass spectrometer was equipped with a Supelcowax 10 fused-silica column (30 m × 0.25 mm; Supelco Inc., Bellefonte, PA) and was temperature programmed from 165 to 265°C at 5°C/min with a 20-min final hold. The GC–MS methodology utilized selected ion monitoring of the appropriate ion masses for the fatty acid in each GC peak. The areas for each of the ion masses monitored were obtained by integration of the peaks. The specific operating conditions and computer-assisted storage and processing of the MS data have been described previously (18,19).

Determination of the weight data for both the isotope-labeled and nonlabeled fatty methyl ester derivatives of the plasma lipid samples was based on the known weight of 17:0 added as an internal standard prior to conversion of the lipid classes to their methyl esters. Response factors were determined by analysis of standard mixtures containing weighed amounts of pure fatty methyl esters purchased from Nu-Chek-Prep Inc. (Elysian, MN) and Applied Science. The accuracy of the GC–MS data was determined by adding known weights of 9*c*-18:1n-9[d2], 20:3n-6[d4], and 20:4n-6[d4] to TG, PL, and CE samples isolated from plasma samples drawn from subjects that were not fed the deuterated fatty ethyl ester mixture. The weight for each of the deuterated fatty esters added was equal to about 0.57% of the total unlabeled fatty acids in the samples. Standard deviations were based on three replicate analyses. The range for the standard deviations for the18:1n-9[d2], 20:3n-6[d4], and 20:4n-6[d4] in the TG, PL, CE spiked samples was 0.005 to 0.008%.

The quantitation of the unlabeled methyl ester data obtained by GC–MS analysis was confirmed by analysis of a subset of samples using a Varian model 3400 gas chromatograph (Varian, Walnut Creek, CA) equipped with a 100 m × 0.25 mm SP2560 fused-silica capillary column (Supelco) and a flame-ionization detector. Operating conditions were: split ratio, 1:100; linear velocity of helium, 21 cm/s; detector and injection temperature, 235°C. Methyl ester GC peaks were identified and quantitation of peak areas was confirmed by analysis of authentic standards and mixtures of known composition.

Statistical analysis and calculations. Data were analyzed with the SAS-PC statistical software package from Statistical Analysis System Institute (Cary, NC). For lipid classes, least square means were compared by analysis of variance and 18:1n-9[d2] vs. 20:3n-6[d4] comparisons used a two-tailed, pairwise *t*-test (20).

The concentrations (μg/mL plasma) of the deuterated fatty acids and their metabolites were calculated by integrating the areas under the time-course curves produced by plotting the deuterated fatty acid data for the eight samples collected between 0 and 72 h, as described previously (21). The values obtained are weighted averages of the eight plasma lipid samples collected over the 72-h time period. Examples of these time-course plots for plasma TG and PL samples from a subject fed the HI20:4 diet are shown in Figures 1 and 2. Plots are included also for percentage enrichment times 100. When the concentration of a lipid class is relatively constant between samples, the curves for percentage enrichment and μg/mL data are generally similar (see Fig. 2), but for TG the concentration of the lipid class varies considerably and percentage enrichment data underestimate the incorporation of the deuterated fatty acids. For this reason, concentration data were used rather than enrichment data to compare the effect of the HI20:4 and LO20:4 diets on incorporation and conversion of the deuterium-labeled fatty acids. The μg/mL data were converted to μmol/mL data and corrected for the differences in the body weight of the subjects and the amounts of

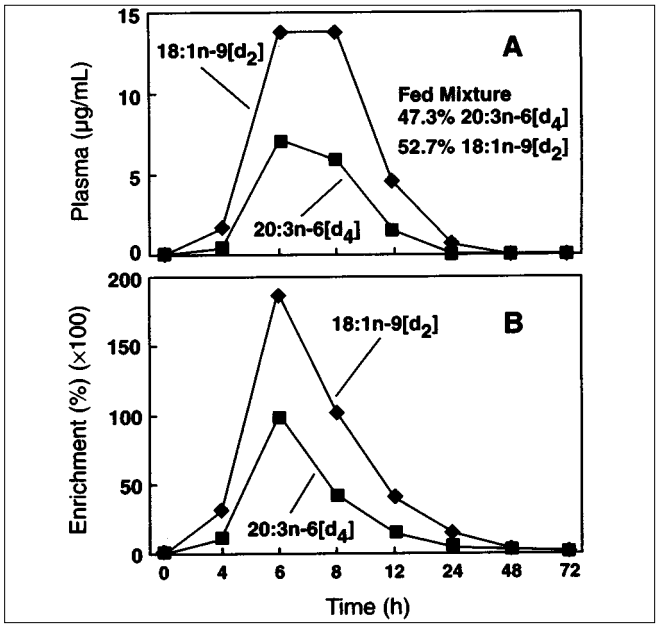

FIG. 1. Time-course plots for incorporation of deuterium-labeled 18:1n-9 and 20:3n-6 into plasma triglyceride samples from subjects fed 1.7 g/d (HI20:4 diet) of 20:4n-6; (A) μmole/mL plasma data; (B) percentage enrichment (×100) of total fatty methyl esters.

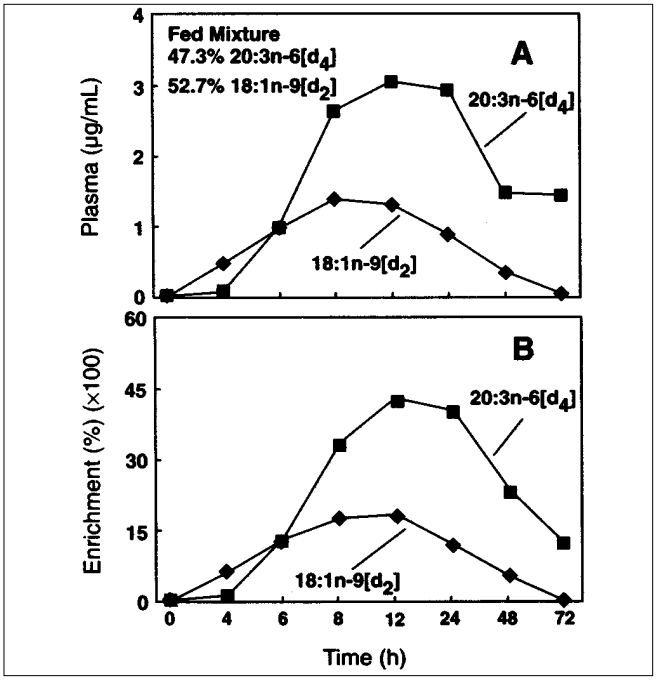

FIG. 2. Time-course plots for incorporation of deuterium-labeled 18:1n-9 and 20:3n-6 into plasma total phospholipid samples from subject fed 1.7 g/d (HI20:4 diet) of 20:4n-6 ; (A) µmole/mL data; (B) percentage enrichment (×100) in total fatty methyl esters.

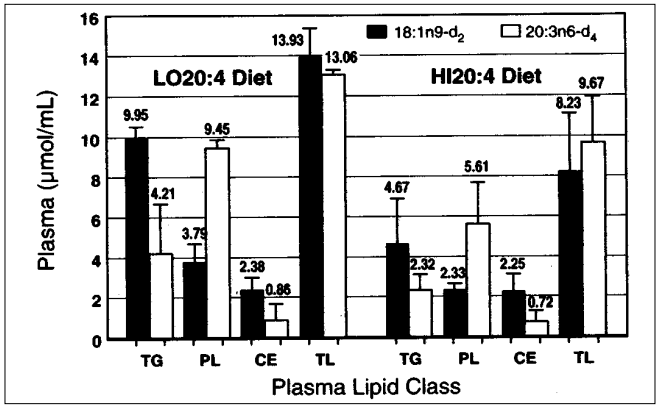

FIG. 3. Concentrations of 18:1n-9[d2] and 20:3n-6[d4] in plasma lipid classes and total lipid from male subjects fed diets containing 0.2 g/d (LO20:4 diet) and 1.7 g/d (HI20:4 diet) of 20:4n-6. Error bars equal standard deviation. Abbreviations: TG, triglyceride; PL, phospholipid; CE, cholesterol ester; TL, total lipid.

deuterated fatty esters fed. This correction was achieved by dividing the µmole/mL plasma data by the mmole of deuterated fatty ester fed per kg of body weight. The total area values obtained from the µmole/mL plasma plots were used to compare the relative uptake and accumulation of deuterated 18:1n-9[d2] and 20:3n-6[d4] fatty acids into plasma lipid fractions and to calculate an estimate of the percentage 20:3n-6[d4] converted to 20:4n-6[d4]. The µmole/mL plasma/mmole deuterated fat fed/kg body weight data was converted to percentage data by dividing the plasma data for individual deuterated fatty acids by the sum of the deuterated fatty acids.

RESULTS

The percentage of deuterated fatty acids in chylomicron TL samples was 57.2 ± 1.77% 18:1n-9[d2] and 42.8 ± 1.77% 20:3n-6[d4] for the subjects fed the HI20:4 diet and 62.7 ± 2.54%18:1n-9[d2] and 37.3 ± 2.54% 20:3n-6[d4] for the subjects fed the LO20:4 diet. The difference between these chylomicron data for the HI20:4 vs. LO20:4 diet groups was not significant at $P < 0.05$. The mean for the chylomicron data from all subjects was 59.9 ± 3.1% for 18:1n-9[d2] and was significantly different ($P < 0.002$) from the mean of 40.1 ± 3.1% for 20:3n-6[d4]. This difference indicates that ethyl 20:3n-6[d4] was about 33% less well absorbed than ethyl 18:1n-9[d2].

The deuterated fatty acid concentrations (µmole/mL) in plasma TL, TG, PL, and CE are plotted in Figure 3. Concentrations of 18:1n-9[d2] and 20:3n-6[d4] in plasma lipid classes for subjects fed the LO20:4n-6 diet were higher than the data for subjects in the HI20:4 diet group, but the data were not significantly different at $P < 0.01$. This lack of a statistically significant effect of diet on the concentrations of the deuterated fatty acids may be the result of a type II error due to the small number of subjects. If a 4-subject per diet group and the same variance as the actual data are assumed, diet would have had a significant effect ($P < 0.01$) on incorporation of 18:1n-9[d2] into TG, PL, and TL but not CE. Also the 20:3n-6[d4] concentrations shown in Figure 3 for plasma TL and PL would have been significantly lower ($P < 0.01$) for the subjects fed the HI20:4 diet compared to the data for the subjects fed the LO20:4 diet. The 20:3n-6[d4] data are consistent with the fatty acid composition data for plasma, red cell, and platelet. Only the total red cell lipids from subjects in the HI20:4 diet group contained a significantly lower ($P < 0.05$) percentage of 20:3n-6 (22). The 20:3n-6[d4] results when combined with the fatty acid composition results indicate that 20:4n-6 dietary supplementation has a limited effect on the incorporation of 20:3n-6 in plasma lipids.

For comparison of 18:1n-9[d2] vs. 20:3n-6[d4] in plasma lipid classes, the concentration of 18:1n-9[d2] was greater than 20:3n-3[d4] in the TG ($P < 0.04$) and CE ($P < 0.012$) fractions, and the concentration of 20:3n-6[d4] in PL was greater ($P < 0.02$) than 18:1n-9[d2]. For TL, the concentrations of 18:1n-9[d2] and 20:3n-6[d4] were not significantly different. The concentrations for 18:1n-9[d2] and 20:3n-6[d4] in plasma TL from subjects fed the LO20:4 diet were significantly higher ($P < 0.005$) than the concentrations of these deuterated fatty acids in TL from subjects fed the HI20:4 diet.

The mean fasting plasma TL concentration of fatty acids was 52% greater (average of 24- and 48-h samples) for subjects from the LO20:4 diet group (3.2 mg/mL) than for subjects from the HI20:4 diet group (2.1 mg/mL). In comparison, the concentrations for the total deuterated fatty acid methyl

esters in plasma TL were 51% greater in subjects from the LO20:4 diet group (27.0 μg/mL) than for the subjects from the HI20:4 diet group (17.9 μg/mL). Since the differences in total plasma lipid concentrations and deuterated fatty acid concentrations were proportional, the difference in the concentration of the deuterated fatty acid concentrations between groups can be explained by the difference between the plasma lipid concentrations of the subjects in the HI20:4 and LO20:4 diet groups.

The μmole/mL data in Figure 3 are replotted as a 100% stacked bar chart in Figure 4 to illustrate the differences between incorporation of the deuterated fatty acids into plasma lipid fractions. The patterns for the concentration data for 18:1n-9[d2] and 20:3n-6[d4] (Fig. 3) and the percentage data (Fig. 4) for the plasma lipid fractions were not influenced by dietary 20:4n-6. The percentage 18:1n-9[d2] in all lipid classes was different from 20:3n-6 at $P < 0.015$. These results suggest that fatty acid structure influenced incorporation and that dietary 20:4n-6 did not influence the selectivity of the acyltransferases for these deuterated fatty acids.

The concentrations of deuterated 20:4n-6 (μmole 20:4n-6[d4]/mL plasma per mmole of 20:3n-6[d4] fed/kg body weight) in plasma TG, PL, CE, and TL are compared in Figure 5 for subjects fed the HI20:4 and LO20:4 diets. For individual lipid classes (TG, PL, CE), the concentrations of 20:4n-6[d4] were two to four times higher in samples from subjects fed the HI20:4 diet than in samples from subjects fed the LO20:4 diet. The differences were significant for PL ($P < 0.03$) and CE ($P < 0.05$) but not for TG. The concentration of 20:4n-6[d4] was about seven times higher in plasma total lipid from subjects fed the HI20:4 diet than in plasma lipids from the subjects fed the LO20:4 diet ($P < 0.001$). In contrast to the concentration data in Figure 3, the difference in the concentration of 20:4n-6[d4] for subjects from the two diet groups was not proportional to the difference in plasma lipid concentration. If the 20:4n-6[d4] data are adjusted for the 50% lower plasma lipid concentration in the subjects fed the HI20:4 diet compared to the subjects fed the LO20:4 diet, the concentration of 20:4n-6[d4] is about 14 times greater for the HI20:4 diet group and reflects the "true" effect of diet.

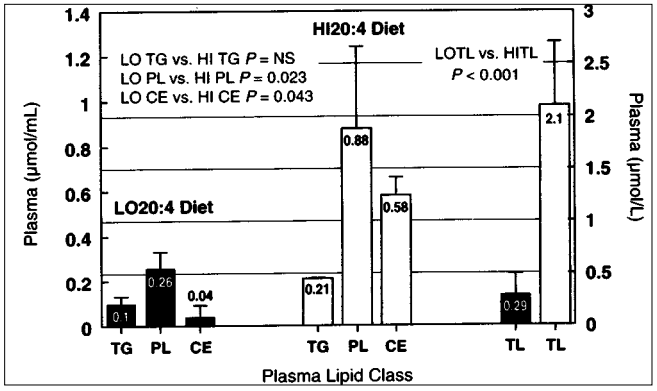

FIG. 5. Concentration of 20:4n-6[d4] in plasma lipids of male subjects fed diets containing 0.2 g/d (LO20:4 diet) and 1.7 g/d (HI20:4 diet) of 20:4n-6. Error bars equal standard deviation. Note that the right side Y-axis scale is used for TL. See Figure 3 for abbreviations.

The effect of diet on the percentage conversion of 20:3n-6[d4] to 20:4n-6[d4] is shown in Figure 6. The percentages for 20:3n-6[d4] conversion were calculated by dividing the 20:4n-6[d4] μmole/mL plasma data by the sum of the 20:3n-6[d4] plus 20:4n-6[d4] μmole/mL plasma data. Percentage conversion based on plasma TL data was 17.7 ± 0.79% for the subjects receiving the HI20:4n-6 diet and 2.1 ± 1.44% for the subjects fed the LO20:4 diet. The 8.3-fold difference in percentage conversion of 20:3n-6[d4] to 20:4n-6[d4] was significant at $P < 0.006$.

The 12-h total PL samples contained the highest concentrations of 20:3n-6[d4]. The percentage enrichment for 20:3n-6[d4] in the 12-h PL samples was 16.8 ± 1.7% for subjects fed the HI20:4 diet and 6.8 ± 1.2% for samples from subjects fed the LO20:4n-6 diet. These percentage enrichment data for 20:3n-6[d4] were significantly different at $P < 0.02$. The 72-h total PL samples contained the highest concentration of 20:4n-6[d4] and the percentage enrichment for

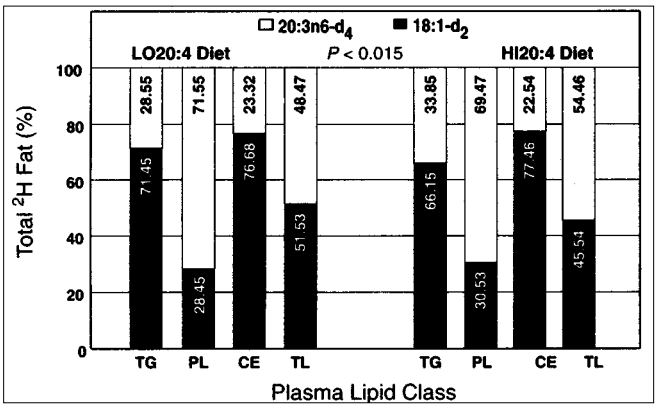

FIG. 4. Percentage of 18:1n-9[d2] and 20:3n-6[d4] in plasma lipid classes and TL from male subjects fed diets containing 0.2 g/d (LO20:4 diet) and 1.7 g/d (HI20:4 diet) of 20:4n-6. See Figure 3 for abbreviations.

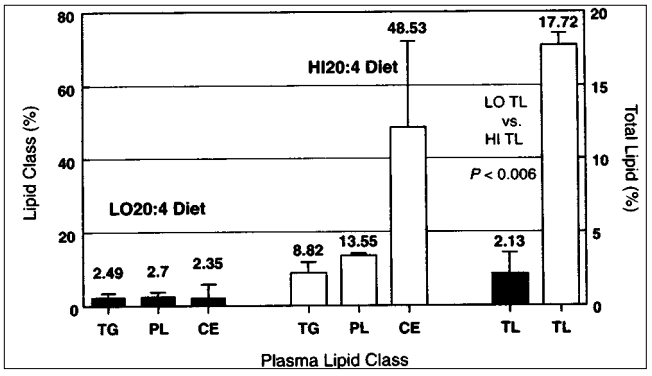

FIG. 6. Percentage conversion of 20:3n-6[d4] to 20:4n-6[d4] in plasma lipid classes and TL from male subjects fed diets containing 0.2 g/d (LO20:4 diet) and 1.7 g/d (HI20:4 diet) of 20:4n-6. Error bars equal standard deviation. See Figure 3 for abbreviations.

20:4n-6[d4] in these 72-h samples was 0.34 ± 0.07% for subjects fed the HI20:4 diet and 0.07 ± 0.054% for subjects fed the LO20:4n-6 diet. The 20:4n-6[d4] percentage enrichment data for the HI20:4 and LO20:4 diet groups were significantly different at $P < 0.05$.

The chain-shortened products (18:3n-6[d4], 18:2n-6[d4], 16:2n-6[d4]) of 20:3n-6[d4] were detected in the plasma lipids of all subjects. The sum of these three products in total plasma lipid from the subjects fed the HI20:4 diet was 0.11 ± 0.01 μmole/mL or 1.1 ± 0.25% of the 20:3n-6[d4]. The total concentration of the chain-shortened products in plasma TL from the subjects fed the LO20:4 diet was 0.37 ± 0.14 or 2.76 ± 0.95% of the 20:3n-6[d4]. The difference between the percent of retroconversion products in plasma TL was not significant. Trace amounts of the 22:3n-6[d4] elongation product of 20:3n-6[d4] and the 22:4n-6[d4] elongation product of 20:4n-6[d4] were detected in 10–15% of the samples. A few samples contained measurable amounts of 22:5n-6[d4], and none of the samples contained detectable amounts of 24:4n-6[d4] or 24:5n-6[d4].

DISCUSSION

Absorption. The absorption of 18- and 20-carbon n-6 and n-3 fatty acids as their ethyl esters, free fatty acids, or TG has been the subject of numerous studies (23). The results from studies that have compared the absorption of unsaturated 18- and 20-carbon fatty acids are not consistent. Studies in humans and rats indicate that 20:5n-3 and 18:1n-9 are equally well absorbed when fed either as their ethyl esters or as TG (24–27) and that 20:4n-6 and 18:1n-9 are equally well absorbed (28), but others have concluded that 20:5n-3 is 30–50% less well absorbed than 18:3n-3 or corn oil (29,30).

The conclusion from this study is that the absorption of ethyl 20:3n-6[d4] was 33% lower than 18:1n-9[d2] which is consistent with results from studies that have compared absorption of 20- to 18-carbon unsaturated fatty acids. Since this conclusion is based on chylomicron TL, there are explanations other than absorption differences that could explain the results. One is that the chylomicron data are a reflection of a more rapid removal of 20:3n-6[d4] than 18:1n-9[d2] from the chylomicron samples. Other possible explanations are that a greater portion of the 20:3n-6[d4] than 18:1n-9[d2] may have been incorporated into intestinally derived very low density lipoprotein TG rather than chylomicron particles or that the absorption of 20:3n-6[d4] was delayed relative to 18:1n-9[d2] in a manner similar to that described for 20:4n-6 (28) .

Incorporation. Owing to the similarity of the patterns for the deuterated 18:1n-9 and 20:3n-6 plasma lipid class data (Figs. 3 and 4), it appears that dietary supplementation with 1.7 g/d of 20:4n-6 for 50 d did not affect the uptake and accumulation of 18:1n-9[d2] relative to 20:3n-6[d4] into plasma lipid classes. For all subjects, the concentration of deuterated fatty acids in the plasma lipid classes was proportional to the concentration of the subject's plasma lipids. Thus the difference shown in Figure 3 for the concentrations of the deuterated fats from the subjects fed the HI20:4 and LO20:4 diets is not believed to be due to the difference in the 20:4n-6 contents of the diets.

The difference in the concentrations of 18:1n-9[d2] and 20:3n-6[d4] in plasma TG, CE, and PL (Fig. 3) and the percentage data shown in Figure 4 illustrate the difference in the selectivity of the TG, PL, and lecithin:cholesterol acyltransferases (LCAT) for 18:1n-9 and 20:3n-6. The difference for accumulation of 18:1n-9[d2] and 20:3n-6n-6[d4] in plasma PL primarily reflects LCAT selectivity and is in contrast to the nonselective incorporation of carbon-14 labeled 18:2n-6 and 20:3n-6 in PL of rat liver microsomes, which reflects acyl CoA cholesterol acyltransferase selectivity (31). Previous results from human subjects fed deuterated 18:1n-9 and 18:2n-6 showed that *in vivo* PL acyltransferase was about 5-fold more selective for 18:2n-6 than 18:1n-9 (32). In this study, the selectivity was 2.44-fold higher for 20:3n-6[d4] than for 18:1n-9[d2]. The combined results from previous studies and this study show that fatty acid chainlength influences n-6 fatty acid accumulation in PL. The high 20:3n-6[d4] to 18:1n-9[d2] ratio of 2.44 in plasma PL is consistent with the recognized high selectivity of PL acyltransferase for n-6 fatty acids. The low 20:3n-6[d4] to 18:1n-9[d2] ratio of 0.34 in plasma CE is consistent with the higher selectivity of LCAT and acyl CoA cholesterol acyltransferase for 18:1n-9 relative to 20:3n-6 and 20:4n-6 (33) and is in contrast to the 18:2n-6 to 18:1n-9 ratio of about 3 in plasma CE for subjects fed deuterated 18:2n-6 and 18:1n-9 (32). The results show that supplementation of diets with 1.5 g/d of 20:4n-6 did not influence significantly the selectivity of the acyltransferases involved in the incorporation of 18:1n-9[d2] and 20:3n-6[d4] into plasma lipids.

Retroconversion. Considerable retroconversion of 20:3n-3 to 18:3n-3 has been observed in mice (18,34), but the retroconversion *in vivo* of 20:3n-6 has not been reported. The approximate 35% retroconversion of deuterated 20:3n-3 observed in mice (18) is in marked contrast to the approximately 2% retroconversion of 20:3n-6[d4] in the human subjects used in this study. The estimate of retroconversion is based on the sum of the deuterated 18-carbon n-6 retroconversion products of 20:3n-6[d4] in total plasma lipid, and it likely underestimates retroconversion because much of the 18:2n-6[d4] and 18:3n-6[d4] chain-shortened products are probably oxidized to acetyl CoA or carbon dioxide. These data indicate also that retroconversion of 20:3n-6[d4] was not significantly influenced by supplementation of the diet with 1.5 g/d of 20:4n-6 for 50 d.

Desaturation. The diet of the LO20:4 subject group contained 0.21 g/d of 20:4n-6 and is probably typical of normal U.S. diets. For these subjects, conversion of 20:3n-6[d4] to 20:4n-6[d4] was low (2.13 ± 1.44%) which is consistent with other reports that humans do not extensively desaturate 20:3n-6 (6,7). The results are also consistent with the low percentage conversion of 18:2n-6 to 20:4n-6 observed in subjects fed deuterated 18:2n-6 (32).

In contrast to the results discussed above, the concentrations of the desaturated product, 20:4n-6[d4], in plasma TL,

TG, PL, and CE were significantly higher for the subjects fed the HI20:4 diet (Fig. 5). Plasma TL percentage conversion data (Fig. 6) suggest that total conversion of 20:3n-6[d4] to 20:4n-6[d4] increased by 8.3-fold when the 20:4n-6 content of the diet was increased to 1.7 g/d. Plasma TL concentration data show a 7.2-fold difference in the amount of 20:4n-6[d4]. The size of this difference for total conversion of 20:3n-6[d4] to 20:4n-6[d4] indicates that a relative small increase (1.5 g/d) in 20:4n-6 intake may markedly influence the 20:3n-6 to 20:4n-6 ratio in tissues and has the potential to produce a change in the amount of eicosanoids synthesized.

The reason the relative pattern of the percentage conversion data in Figure 6 for the individual lipid classes is different from the pattern for the concentration data in Figure 5 is that calculation of the estimated percentage conversion is dependent on the amount of both 20:4n-6[d4] and 20:3n-6[d4]. For example, in Figure 6 the percentage conversion for CE is larger than for PL because the concentration of 20:3n-6[d4] in CE is small rather than because 20:4n-6[d4] is large.

For subjects fed the LO20:4 diet, the μmole/mL plasma data and percentage data in Figures 5 and 6 are consistent with the low conversion of 20:3n-6 observed with rabbit and hamster models relative to rat and mouse models which have higher Δ5-desaturase activity than rabbits, hamsters, and humans (6). Comparison of the HI20:4 and LO20:4 diet data indicates that, in humans, conversion of 20:3n-6[d4] to 20:4n-6[d4] by Δ5 desaturation was stimulated by dietary supplementation with 1.5 g/d of 20:4n-6 for 50 d. These *in vivo* data in humans are supported by results from cell culture studies that report a 40–50% higher conversion of 18:2n-6[^{14}C] to 20:4n-6[^{14}C] with cells incubated in media containing added 20:4n-6 (35–37).

Classical enzyme kinetic theory suggests that dietary 20:4n-6 intake should influence the metabolism of 20:3n-6 due to the substrate–product relationship. Thus classical theory would predict that an increase in dietary 20:4n-6 should reduce conversion of 20:3n-6[d4] to 20:4n-6[d4] because of product feedback inhibition. Since stimulation of 20:3n-6 conversion to 20:4n-6 by dietary 20:4n-6 supplementation cannot be explained by simple enzyme kinetic data, our results suggest that conversion *in vivo* of 20:3n-6 to 20:4n-6 is controlled by mechanisms other than or in addition to the rate of Δ5 desaturation.

It is known that Δ5 desaturase prefers the CoA thioester derivative of 20:3n-6 as a substrate rather than 1-acyl-2-eicosatrienoyl-*sn*-glycerol-3-phosphorylcholine (38). Also, most of the 20:3n-6 in tissue lipids is in the 2-acyl position of PL (1,5,32), and 20:3n-6, like 20:4n-6, is conserved by an efficient deacylation–reacylation mechanism that rapidly reincorporates these fatty acids into PL after hydrolysis (39). The combined effect of these observations suggests that most of the 20:3n-6 and 20:4n-6 in tissue lipids are "locked" into PL structures, and only small amounts are available as substrates for other reactions.

Thus, we propose as a working hypothesis that an increase in dietary 20:4n-6 increases the PL deacylation–reacylation reaction rates or the turnover rate for 20:3n-6 and 20:4n-6. A higher turnover rate would increase the availability in the free fatty acid pool of 20:3n-6[d4] that had been incorporated into PL and thus allow formation of its CoA derivative and its subsequent desaturation. This sequence could explain the increased conversion of 20:3n-6[d4] to 20:4n-6[d4] that was observed. The possibility that dietary 20:4n-6 greatly increased the direct desaturation of the 20:3n-6[d4] incorporated into the PL pool is unlikely because supplementation with 20:4n-6 did not have a significant effect on the μmole of 20:3n-6[d4] incorporated per mg of total PL. In contrast, the percentage enrichment for 20:3n-6[d4] in plasma total PL for the HI20:4 diet group subjects was 2.5-fold higher than for the LO20:4 diet group. This difference indicates that dietary supplementation with 20:4n-6 increased the turnover rate of 20:3n-6 in the PL pool. The percentage enrichment for 20:4n-6[d4] was 5-fold higher for samples from subjects fed the HI20:4 diet which is additional evidence in support of a higher turnover rate.

The implication of the results from this study is that, if dietary 20:4n-6 intake is low, dietary 20:3n-6 should increase the 20:3n-6 to 20:4n-6 ratio in tissue lipids because the 20:3n-6 levels would increase, but the 20:4n-6 levels would not greatly increase. This change in the 20:3n-6 to 20:4n-6 ratio might in turn influence the production or balance of eicosanoids synthesized from these fatty acids. If the dietary intake of 20:4n-6 is high, supplementation with 20:3n-6 could paradoxically lower rather than increase the 20:3n-6 to 20:4n-6 ratio owing to enhanced conversion of 20:3n-6 to 20:4n-6. However, the effect of dietary supplementation with large amounts of 20:3n-6 on 20:3n-6 conversion to 20:4n-6 and on 20:4n-6 metabolism is not known. Nevertheless, the results from this study on the incorporation, absorption, and conversion of 20:3n-6[d4] to 20:4n-6[d4] illustrate that there is still much that needs to be learned about the effect of dietary n-6 fatty acids and the regulation of their metabolism.

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Effects of Dietary Arachidonic Acid on Human Immune Response

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ABSTRACT: Arachidonic acid (AA) is a precursor of eicosanoids, which influence human health and the *in vitro* activity of immune cells. We therefore examined the effects of dietary AA on the immune response (IR) of 10 healthy men living at our metabolic suite for 130 d. All subjects were fed a basal diet containing 27 energy percentage (en%) fat, 57 en% carbohydrate, and 16 en% protein (AA, 200 mg/d) for the first and last 15 d of the study. Additional AA (1.5 g/d) was incorporated into the diet of six men from day 16 to 65 while the remaining four subjects continued to eat the basal diet. The diets of the two groups were crossed-over from day 66 to 115. *In vitro* indexes of IR were examined using the blood samples drawn on days 15, 58, 65, 108, 115, and 127. The subjects were immunized with the measles/mumps/rubella vaccine on day 35 and with the influenza vaccine on day 92. Dietary AA did not influence many indexes of IR (peripheral blood mononuclear cell proliferation in response to phytohemagglutinin, Concanavalin A, pokeweed, measles/mumps/rubella, and influenza vaccines prior to immunization, and natural killer cell activity). The post-immunization proliferation in response to influenza vaccine was about fourfold higher in the group receiving high-AA diet compared to the group receiving low-AA diet ($P = 0.02$). Analysis of variance of the data pooled from both groups showed that the number of circulating granulocytes was significantly ($P = 0.03$) more when the subjects were fed the high-AA diet than when they were fed the low-AA diet. The small increases in granulocyte count and the *in vitro* proliferation in response to influenza vaccine caused by dietary AA may not be of clinical significance. However, the lack of any adverse effects on IR indicates that supplementation with AA may be done safely when needed for other health reasons.

Lipids 32, 449–456 (1997).

Both the concentration and type of dietary fat have been reported to alter immune response (IR) in several human studies. Decreasing the amount of fat in the diet enhanced several indexes of IR, and the converse was true when fat intake was increased (1–3). Supplementing diets with n-3 polyunsaturated fatty acids (PUFA) from flaxseed or fish oils inhibited several indexes of IR (4–7), while a moderate increase in the intake of n-6 PUFA inhibited IR in some but not in other studies(1–3,8). The source of n-6 PUFA used in the above studies was oils rich in linoleic acid (LA), which can be converted to arachidonic acid (AA) in healthy individuals through desaturation and chain elongation. LA can have some direct effects on metabolic functions, but most of its effects are due to its conversion to AA. AA is one of the major fatty acids of the membranes and is a precursor for eicosanoids. Dietary AA is not required for humans, but it has been reported to alter tissue fatty acid composition and the eicosanoids produced in humans (9,10). Results from a study with Syrian hamsters indicate that dietary AA was metabolized differently from the endogenously synthesized AA (11). Addition of AA or its metabolites to cultured immune cells altered their activity and the eicosanoids produced *in vitro* (12–17). These studies suggest that AA and its metabolites may play an important role in modulating IR, and that the metabolism of AA may be altered depending on its source.

There is currently no recommended daily allowance (RDA) for AA or other fatty acids for humans. Diets rich in eggs and organ meats are high in AA. The estimates for the daily intake of AA in the United States for adults consuming eggs and meats range from 200–1000 mg with an average of about 500 mg (18,19). Others, however, have estimated the daily intakes of AA from Western diets to range from 100–500 mg (20–23).

No human or animal studies have examined the effects of dietary AA on IR. The purpose of this study was to examine the effects of dietary AA on several indexes of human IR, particularly the adverse consequences from the increased intake of AA. Considering the safety of the subjects and the minimum amount of AA needed to perturb the tissue fatty acid composition, we modified the diets of 10 healthy men to contain additional AA (1.5 g/d for 50 d) using a crossover design. The indexes of IR examined in this study were those that have previously been shown to be affected by the amount and type of dietary fat; these included lymphocyte

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Abbreviations: AA, arachidonic acid; CD, cluster designation; Con A, concanavalin A; DTH, delayed-type hypersensitivity skin response; en%, energy percentage; FBS, fetal bovine serum; II-2, interleukin-2; INF, interferon; LA, linoleic acid; MMR, measles/mumps/rubella; MP, metabolic period; NK, natural killer; PGE, prostaglandin E; LTB_4, leukotriene B_4; PBMNC, peripheral blood mononuclear cells; PUFA, polyunsaturated fatty acids; PHA, phytohemagglutinin; RDA, recommended daily allowance.

proliferation, phenotypic analysis for B and T cells, helper, suppressor, cytotoxic, and natural killer (NK) cells, NK cell activity, and *in vivo* delayed-type hypersensitivity (DTH) skin response.

MATERIALS AND METHODS

Subjects, protocol, and diets. The research protocol and informed consent form were approved by the Institutional Review Board of the University of California, Davis, and by the Human Studies Review Committee of the Agricultural Research Service, U.S. Department of Agriculture (Washington, D.C.). Twelve healthy men (ages 20–38 yr) were selected to participate in the study after medical and physiological screening. Only subjects that were nonsmokers, had no history of alcohol or drug abuse, had moderate physical activity and body weight within −10 to +20% of ideal body weight, and had hematological and chemical measurements within normal ranges were included in the study. Data from two subjects, who did not complete the study, were not included.

The participants lived and ate all meals at the Western Human Nutrition Research Center (WHNRC) (San Francisco, CA) for 130 d (August 14–December 22, 1994). All subjects consumed the basal/reference (low-AA) diet during the first 15 d (metabolic period 1, MP1). For the next 50 d (MP2), six subjects (Group 1) consumed the high-AA diet and the other four (Group 2) continued to consume the basal diet. Diets of the two groups were switched on day 66, and the new diets were fed between days 66 and 115 (MP3). Both groups were fed the low-AA diet between days 116 and 130 (MP4). Body weights of the subjects were maintained constant throughout the study by adjusting the caloric intake and by maintaining a constant physical activity. All subjects went for two two-mile walks every day. Blood samples from the subjects were collected on days 16, 58, 65, 108, 115, and 127 to study *in vitro* indexes of IR. All subjects were immunized with measles/mumps/rubella (MMR) vaccine on day 35 and with influenza vaccine on day 92. Antigens for DTH skin response were injected into all subjects on days 65 and 115.

Both diets were composed of natural foods and were adequate in all nutrients. Diets were fed on a 5-d rotating menu with three meals and two snacks every day. The planned percentage of energy from carbohydrate, fat, and protein in both diets was 55, 30, and 15% respectively. The 30% calories from fat in the basal diet were intended to come 10% from each of the saturated, monounsaturated, and n-6 PUFA. On an average, the basal diet provided 200 mg AA and 250 mg cholesterol every day. The calculated level of vitamin E in both diets was at twice the RDA for this nutrient. ARASCO® oil, a gift from Martek Biosciences Corporation (Columbia, MD), was the source of additional AA for the high-AA diet. This oil is purified from algae and contains fatty acids as natural triglycerides. Its fatty acid composition by weight percentage is AA 50, palmitic acid 9, stearic acid 14, oleic acid 9, linoleic acid 6, γ-linolenic acid 4, homo-γ-linolenic acid 3, and other fatty acids 5. AA (1.5 g/d from 3 g of ARASCO® oil) was incorporated into the diets by replacing an equivalent amount of the monounsaturated fat from the basal diet. ARASCO® oil was kept at −20°C under nitrogen until ready to be served in cold yogurts or salad dressings. Other details of the study design are given in a preceding paper in this journal volume (24).

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

Blood cell count and lymphocyte phenotypic analysis. For each blood draw, a complete and differential cell count was performed by using a Serono Baker Automated system (model 9000 diff; Allentown, PA). Phenotypic analysis of the various lymphocyte subsets was done using Becton-Dickinson FACStar flow cytometer (San Jose, CA). Whole blood collected in EDTA-containing vacutainer tubes was transferred into tubes containing fluorochrome-labeled monoclonal antibodies against specific cluster differentiation (CD) antigens. After incubation for 30 min, the red cells were lysed with formaldehyde and removed by washing the leukocytes twice with Dulbecco's phosphate buffered saline without calcium and magnesium. The stained lymphocytes were fixed in 1% paraformaldehyde prior to analysis with the flow cytometer. The percentage of lymphocytes of a given phenotype was determined from a total of 10,000 lymphocytes counted.

Isolation and culture of peripheral blood mononuclear cells (PBMNC). The PBMNC were isolated using Histopaque-1077 as previously reported (25). The culture medium used was RPMI-1640, (Gibco, Grand Island, NY) containing 10% autologous serum and L-glutamine (2 mmol/L), penicillin (100 KU/L), streptomycin (100 mg/L), and gentamicin (20 mg/L). One hundred μL of the culture medium containing 1×10^5 PBMNC was inoculated in each well of a 96-well flat-bottom culture plate. An additional 100 μL of the culture medium with or without the mitogens was added to each well. The mitogens used were phytohemagglutinin (PHA), Concanavalin A (Con A), and pokeweed (all from Sigma Chemical Co, St. Louis, MO). Each mitogen was used at two concentrations; final concentrations (mg/L) in the culture media were 10 and 20 for both PHA and Con A, and 0.005 and 0.05 for pokeweed. Separate cultures of PBMNC were also stimulated with influenza and MMR vaccines at three different concentrations (1:250, 1:500, 1:1000 dilutions). Trivalent influenza virus vaccine (Fluzone, 1993–1994 Formula) was from Connaught Laboratories Inc. (Swiftwater, PA) and the MMR live vaccine, MSD, was from Merck, Sharp & Dohme (West Point, PA). PBMNC stimulated with mitogens were cultured for 72 h and those stimulated by antigens for 120 h; [^{3}H] thymidine, 37 K Bq, in 50 μL, was added to each well during the last 24 h. PBMNC were collected on filter strips and the radioactivity was determined using a Packard β-gas counter. The [^{3}H]thymidine incorporation into cellular DNA (Bq/1000 cell) was used as the index of PBMNC proliferation.

Determination of NK cell activity. A fraction of the PBMNC isolated above was suspended in serum-free RPMI to a final concentration of $3–5 \times 10^6$/mL to be fractionated for determining NK cell activity. PBMNC suspension (3 mL) was mixed with 7 mL of RPMI 1640 containing 10% fetal bovine serum (FBS), and incubated in T75 culture flasks for 1 h at 37°C in a 5% CO_2 incubator. Parallel flasks with the addition of interleukin-2 (IL-2) (30,000 IU/L) and interferon γ (INF_γ, $I \times 10^6$ IU/L) to stimulate NK cell activity were also prepared. After 1 h incubation, the flasks were gently shaken and the nonadherent cells were transferred into 15-mL conical tubes and centrifuged for 5 min at $500 \times g$. The pellet was resuspended in 1 mL RPMI containing 10% FBS, and cell concentration determined, and adjusted to 6.6×10^6/mL. ^{51}Cr-labeled K-562 cells were used as the target cells, and their concentration was determined by using the Elzone Particle Counter (Particle Data Inc., Los Angeles, CA). The cell concentration was adjusted to 2×10^5 cells/mL with RPMI 1640 containing 10% FBS. Cell suspension (50 μL) containing 10,000 of K-562 cells was added to each well of the 96-well round-bottom plates. The nonadherent PBMNC isolated above were then added at concentrations of $(0.50, 0.25, 0.125, 0.063, 0.031) \times 10^6$ to each well of the 96-well plate to give effector/target cell ratios of 50:1, 25:1, 12.5:1, 6.2:1, and 3.1:1. Six wells were used for each effector cell concentration and for the spontaneous and maximum release (caused by 3% centrimide) of ^{51}Cr.

Plates were incubated for 4 h at 37°C in 5% CO_2 and then centrifuged for 10 min at $200 \times g$. The supernatant (100 μL) was removed from each well into vials and the radioactivity released was determined by using a Packard gamma counter (Hewlett-Packard, Palo Alto, CA). Percentage lysis was calculated in Equation 1.

$$\% \text{ lysis} = \frac{(\text{experimental cpm} - \text{spontaneous cpm})}{(\text{maximum cpm} - \text{spontaneous cpm})} \times 100 \quad [1]$$

DTH. DTH response to seven recall antigens was assayed by intradermally injecting 0.1 mL of each antigen solution into the forearm. The antigens used were tuberculin purified-protein derivative (one international test unit), mumps (four complement-fixing test units), tetanus toxoid (1:100, vol/vol dilution of a solution containing four flocculation units/0.5 mL), candida (1:100, vol/vol dilution), trichophyton (1:30, vol/vol dilution), streptokinase streptase (100 KU/L), and coccidioidin (bioequivalent to U.S. reference coccidioidin 1:100; provided by the Office of Biologics, Food and Drug Administration, Washington, D.C.). The antigens were diluted with a diluent containing, per liter, 3 mL normal human serum and 9 g sodium chloride. Tuberculin purified-protein derivative, mumps, and tetanus toxoid were supplied by Connaught Laboratories Inc. (Swiftwater, PA). Candida (Dermatophyton O), trichophyton, and the antigen diluent were obtained from Hollister Stier (Spokane, WA). Streptokinase streptase, and coccidioidin were purchased from Behringwereke Ag (Marburg/Lahn, Germany) and Berkeley Biologicals (Berkeley, CA), respectively. Response to these antigens was determined by measuring mean induration diameters (mm) at 48 and 72 h after injections. Induration diameters with less than 4 mm were scored negative. Data are reported as the sum of induration diameters for all positive responses (induration score) and the number of positive responses to the seven antigens (antigen score).

Data analysis. The data were analyzed with analysis of variance by using SAS/STAT PROC GLM (26). The crossover design model included effects of order, subject (order), period, and diet, using subject (order) as an error term for order. If the period effects were not significant, the data from two groups were pooled (e.g., leukocyte counts), otherwise analyzed separately (e.g., DTH skin response, NK cell activity, and PBMNC proliferation in response to mitogens). The significance of the differences between the effects of the two diets was assessed from the *P* values for the diet main effects. The data from the two groups were analyzed separately using a paired *t*-test when the treatment was given only during one period, such as immunization with MMR or influenza vaccines. Changes in the variables examined are considered significant for $P < 0.05$ or otherwise stated.

RESULTS

As stated in the Materials and Methods section, the 10 subjects included in this study were divided into two groups. The high-AA diet was fed to subjects in Group 1 ($n = 6$) from study days 16 to 65, and to those in Group 2 ($n = 4$) from study days 66 to 115. The mean ± SEM for age (yr), weight (kg), height (cm), and body mass index (kg/m^2) for subjects in Groups 1 and 2 were 31.2 ± 3.2 and 32.2 ± 2.9, 73.8 ± 2.4 and 71.0 ± 5.4, 177.4 ± 3.6 and 175.5 ± 1.8, 23.8 ± 1.8 and 23.0 ± 1.3, respectively. None of these parameters was statistically different between the two groups.

Dietary intake data prior to entering the study were not collected; however, all subjects consumed a typical American diet, which provides 35–40% calories from fat. The mean daily intake of nutrients from the low- and high-AA diets are shown in Table 1. These intakes are based on an average daily intake of 2800 kcal, which did not differ between the two diets or two groups. The proximate analysis for the macronutrients showed that the energy percentage (en%) from the protein, fat, and carbohydrate were not different between the two diets. These analyzed values were 27, 57, and 16 en% from fat, carbohydrate, and protein, respectively, which were close to the planned values of 30, 55, and 15% respectively.

The weight percentage fatty acid compositions of the two diets are shown in Table 2. While there are minor differences in the concentration of other fatty acids between the two diets, the major difference is in the concentration of AA, which is nine times higher in the AA diet than in the basal diet. The high-AA diet contained about 2% less oleic acid than that found in the low-AA diet.

White blood cell counts and lymphocyte subsets. The data

TABLE 1
Mean Daily Nutrient Intake[a]

Nutrient	Low-AA diet	High-AA diet
Protein		
(g)	104.6 ± 3.1	106.0 ± 5.3
(% of energy)	15.8 ± 0.5	15.2 ± 0.8
Total fats		
(g)	91.6 ± 4.7	85.2 ± 6.7
(% of energy)	27.1 ± 1.5	27.9 ± 2.2
Carbohydrates		
(g)	388.7 ± 9.8	401.8 ± 11.1
(% of energy)	57.1 ± 1.4	57.0 ± 1.6
Ash (g)	17.0 ± 1.3	19.5 ± 1.6
Copper (mg)	1.27	1.26
Iron (mg)	23.5	22.1

[a]Mean ± SEM (n = 5). Proximate analysis was performed on a composite sample of each diet prepared for each day of the 5-d rotating menu. For copper and iron, analysis was done on pooled samples from the individual composites; AA, arachidonic acid.

for leukocyte counts (Fig. 1) and lymphocyte subsets (not shown) could be pooled from all 10 subjects to determine the effects of the two diets, since the period effects (carryover) for these variables were not significant. The rank transformation was used to stabilize the subject variances among groups and periods, so the difference is in terms of means of ranks. For the diet effect, the crossover analysis of variance model tested the difference between the average of the high-AA diet and the low AA-diet. Figure 1 shows that compared to the baseline values the number of circulating leukocytes increased during the study in both groups, but the difference between the high- and low-AA diets did not attain statistical significance (P = 0.1). This increase in leukocyte numbers was primarily due to the increase in circulating granulocytes (Fig. 1), and the number of circulating monocytes and lymphocytes did not change (not shown). The increase in the number of circulating granulocytes was significantly (P = 0.03) higher when the subjects were fed the high-AA diet than when they were fed the low-AA diet.

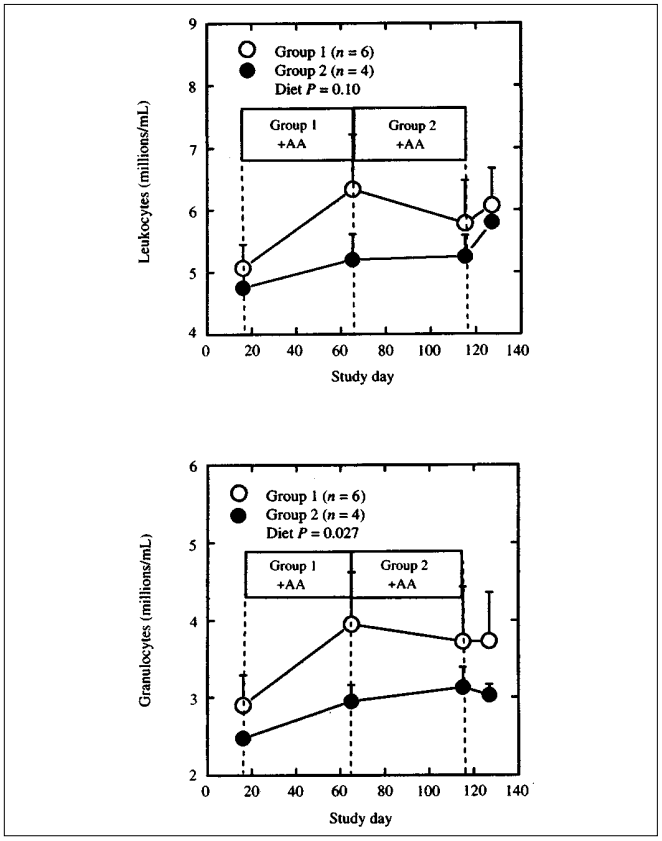

FIG. 1. Effect of low- and high-arachidonic acid-(AA) diets on the number of circulating leukocytes and granulocytes. Mean ± SEM. Group 1 was fed high-AA diet between days 16 and 65 and group 2 between days 66 and 115; low-AA diet was fed for all other days. Crossover analysis of variance model for the rank transformed data showed a significant (P = 0.027) difference between the number of circulating granulocytes when subjects were fed the high- vs. the low-AA diets, while the increase in the number of leukocytes was not significant (P = 0.1).

TABLE 2
Fatty Acid Composition (wt%) of Experimental Diets[a]

Fatty acid	Low-AA diet	High-AA diet
12:0	0.73 ± 0.18	0.68 ± 0.10
14.0	2.43 ± 0.65	2.38 ± 0.60
14:1n-5	n.d.	0.14 ± 0.05
15:0	0.30 ± 0.07	0.29 ± 0.07
16:0	15.11 ± 1.53	14.98 ± 1.50
16:1n-9	0.83 ± 0.13	0.80 ± 0.13
17:0	0.32 ± 0.04	0.31 ± 0.03
18:1n-9 DMA	0.24 ± 0.04	0.23 ± 0.03
18:0	7.43 ± 0.71	7.47 ± 0.64
18:1n-7 *trans*	6.14 ± 0.85	5.92 ± 0.83
18:1n-9	34.64 ± 1.46	31.94 ± 1.40
18:1n-7	1.50 ± 0.07	1.42 ± 0.07
18:1n-5	1.45 ± 0.30	1.44 ± 0.29
18:2 *trans-trans*	0.50 ± 0.06	0.55 ± 0.07
18:2n-6	23.77 ± 2.01	24.07 ± 1.86
18:3n-6	0.37 ± 0.01	0.39 ± 0.01
18:3n-3	1.97 ± 0.29	1.98 ± 0.27
20:1n-11	0.25 ± 0.02	0.25 ± 0.01
22:0	0.26 ± 0.01	0.30 ± 0.02
20:4n-6	0.24 ± 0.02	2.19 ± 0.11
Total	98.50 ± 0.03	97.72 ± 0.17
Unknowns	1.50 ± 0.03	2.28 ± 0.17

[a]Mean ± SEM (n = 5); n.d. = none detected; DMA = dimethyl acetal of (9*Z*)-octadecenal. See Table 1 for abbreviation.

Proliferation of PBMNC cultured with antigens and mitogens. Proliferation of PBMNC cultured with three different dilutions (1:250, 1:500, and 1:1000) of influenza vaccine are shown in Figure 2. This figure shows that prior to *in vivo* immunization the proliferation of PBMNC cultured with all three dilutions of the vaccine did not differ between the two groups, and that it significantly increased in both groups following *in vivo* immunization on day 92. The increases in PBMNC proliferation were, however, different between the two groups. For Group 1 consuming the low-AA diet, the maximum increase in proliferation caused by the influenza vaccine was about 100%, with P values of 0.355, 0.047, and

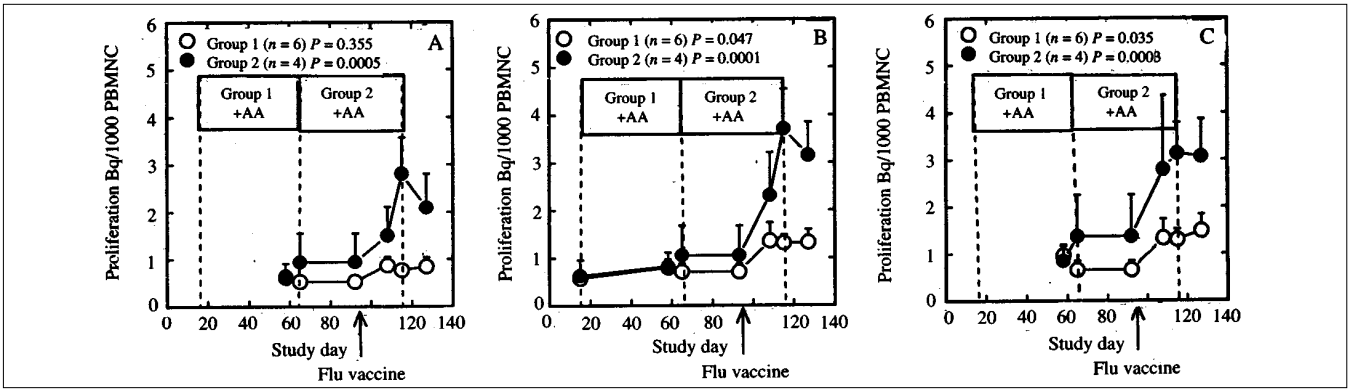

FIG. 2. Effect of low- and high-AA diets on the proliferation of peripheral blood mononuclear cells (PBMNC) cultured with influenza vaccine. X ± SEM. Diet schedule same as in the legend to Figure 1. Analysis of variance showed no difference between the two groups prior to the *in vivo* immunization on day 92. After the *in vivo* immunization, proliferation increased in both groups, and the increase was significantly ($P = 0.02$) higher in group 2 than in group 1. See Figures 1 and 2 for other abbreviations. Dilutions: (A) 1:1000 (B) 1:500 (C) 1:250.

0.035 at the vaccine dilutions of 1:1000, 1:500, and 1:250, respectively. For Group 2, which was consuming the high-AA diet, the maximum increase in PBMNC proliferation in response to influenza vaccine was about 400% and the *P* values for the three dilutions of the vaccine were 0.0005, 0.0001, and 0.0003. The difference between the two groups in PBMNC proliferation *in vitro* after *in vivo* immunization with the influenza vaccine was significant ($P = 0.02$). The three dilutions of MMR vaccine that were tested (1:1000, 1:500, and 1:250) failed to stimulate *in vitro* PBMNC proliferation above the basal level both at pre- and postimmunization with this vaccine (not shown).

Figure 3 contains the data regarding proliferation of PBMNC cultured with three different mitogens: PHA and Con A (20 mg/L) and pokeweed (0.05 mg/L). These concentrations caused the maximum proliferation of PBMNC for each mitogen. This figure shows that PBMNC proliferation in response to PHA and Con A was different in the two groups at the start of the study; however, by the end of the study, the proliferation levels in the two groups were not different. There was no effect of dietary AA on PBMNC proliferation in response to all three mitogens in both groups. The basal diet, however, caused significant increases in PBMNC proliferation in response to Con A and pokeweed in both groups and PHA in Group 2 only. Results obtained with the second concentration of these mitogens (10 mg/L for PHA and Con A, and 0.005 mg/L for pokeweed, not shown) were similar to those shown in Figure 3. The proliferation of PBMNC cultured without mitogens was about 1% of that obtained with pokeweed (not shown). No effect of AA or basal diet could be detected on the proliferation of PBMNC cultured without mitogens.

DTH. All 10 subjects responded to tetanus and mumps, six to candida, three to trichophyton, and two to each of streptokinase, tuberculin, and coccidioidin.The cumulative induration scores determined 48 h after the application of DTH antigens for Group 1 at day 65 and day 115 were 20.1 ± 4.2 and 46.6 ± 13.3 mm (mean ± SEM), and for Group 2 were 31.9 ± 6.1 and 54.2 ± 10.1 mm. The corresponding antigen scores (number of antigens testing positive) for Group 1 were 2.33 ± 0.42 and

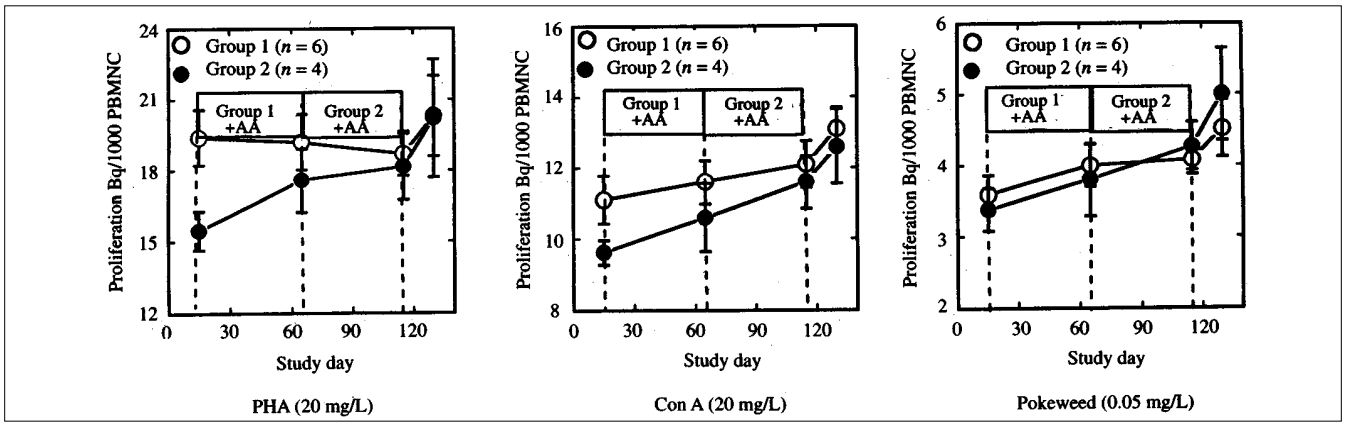

FIG. 3. Effect of low- and high-AA diets on the proliferation of PBMNC cultured with mitogens. X ± SEM. Diet schedule same as given in the legend to Figure 1. Analysis of variance showed no effect of AA on all variables included in this figure; however, there was a significant ($P < 0.05$) increase with time for all variables except the phytohemagglutinin response in group 1.

3.17 ± 0.6, and for Group 2 were 3.25 ± 0.48 and 3.75 ± 0.85, respectively. In both groups, induration and antigen scores on day 115 were higher than those on day 65. This was perhaps caused by the low-fat basal diet. There was no difference in the induration and antigen scores between the subjects fed the high- or low-AA diet. Induration results obtained at 72 h were similar to those at 48 h in Group 1, but in Group 2 the difference between the induration found on days 65 and 115 was not significant (not shown).

NK cell activity. In both groups of subjects, the basal level (unstimulated) NK cell activities determined at effector/target ratios of 50:1, 25:1, 12.5, 6.25:1, and 3.1:1 did not change during the study from their values at study day 16 (not shown). These data suggest that NK cell activities were not affected by the low- and high-AA diets. Incubation of NK cells *in vitro* with IL-2 and INFγ caused a 30–35% increase in the percent lysis caused by them at all effector/ target cell ratios when compared to the corresponding percent lysis caused by nonstimulated cells. However, again there was no effect of the low- or high-AA diet on NK cell activity.

DISCUSSION

In this study, we attempted to define the effect of dietary AA on human IR by feeding a natural triglyceride of AA. We had intended to feed AA at 3.0 g/d for 50 d, but in order to minimize health risk to the subjects, the Human Use Committee approved only 1.5 g/d. The study was designed assuming that there will be no period effect (effect of basal diet or that of the residual AA fed in the previous period), and that data from all subjects could be pooled. However, the statistical analysis of the data showed that for several of the variables there were significant period effects, for which the data from two groups had to be analyzed separately. Our results show that dietary AA significantly increased the number of circulating granulocytes, and the secondary response to influenza vaccine. Several other indexes of IR, including DTH skin response, PBMNC proliferation in response to PHA, Con A, and pokeweed, NK cell activity, number of circulating lymphocytes bearing markers for B and T cells, helper, suppressor, cytotoxic, and NK cells were not affected by the additional intake of AA. The cumulative effect of AA used in this study may actually be lower than that of AA-rich diets based on eggs and meats eaten for years. Lymphocyte proliferation in response to mitogens, and the number of circulating leukocytes were increased by both the low- and high-AA diets when compared to the corresponding values at the start of the study (day 16). The DTH response at day 115 was also significantly higher than the corresponding values on day 65 in both groups of subjects. Some of these changes may be due to the low-fat nutritionally balanced basal diet, as we previously observed (1,2).

We are not aware of any human or animal studies where AA consumption has been reported to increase the number of circulating granulocytes (neutrophils). However, injection of AA metabolites [prostaglandin E (PGEs) and leukotrienes] into human skin induced a dose-dependent erythema by increasing the supply of blood to the inflamed site (27–29). The circulating neutrophils are thought to be in dynamic equilibrium with their marginating pools. Neutropenia can be caused by inflammatory factors like endotoxin and C5a (30,31), and neutrophilia can be caused by exercise or adrenaline infusion (32). Leukotriene B_4 (LTB_4) is a chemoattractant for neutrophils, and addition of AA *in vitro* has been found to enhance neutrophil chemotaxis and diapedesis (33). The increase in the consumption of AA in our study may have increased the circulating levels of LTB_4 and other chemoattractant AA metabolites, which may have led to the increase in the number of circulating neutrophils. There also appears to be an AA-independent increase in the number of circulating neutrophils, which was the difference in the values at the end of the low-AA diet and at the end of the stablization period. This increase could have been caused by several factors, including a latent infection, confinement stress, increased exercise, and the effects of dietary components other than AA. We believe it was most likely due to the daily four-mile walks, as exercise has been reported to increase the number of circulating neutrophils (32).

Prior to immunization with the influenza vaccine, the proliferation of PBMNC stimulated with the same vaccine *in vitro* was comparable between the subjects fed the low- and high-AA diets (Fig. 2). However, following *in vivo* immunization, the *in vitro* proliferation in response to this vaccine was significantly higher in the group fed the high-AA diet than in the group fed the low-AA diet (Fig. 2). Thus, AA effect on PBMNC proliferation in response to influenza vaccine seems to be on the secondary rather than on the primary response of lymphocytes. Whether the increased lymphocyte proliferation will translate into increased antibody production cannot be determined from our data because we did not examine *in vitro* cell differentiation or antibody production. Enhancement of secondary antibody response to keyhole limpet hemocyanin has been reported by the addition of AA to rabbit popliteal lymph node cells *in vitro* (34). Similarly, in humans the secondary antibody response to influenza A-victoria was significantly enhanced by indomethacin, an inhibitor of AA metabolism through the cyclooxygenase pathway (35). These results imply that the secondary antibody response was enhanced by the increased production of lipoxygenase products. We believe the difference in PBMNC proliferation in response to influenza vaccine between the two groups was due to stimulation of this response by AA in Group 2. However, it is also possible that it is due to an inhibition by the residual AA in Group 1. Our study design cannot distinguish between these two possibilities.

The effect of AA on PBMNC proliferation was specific for influenza vaccine, because no stimulation was found when PBMNC were stimulated with MMR, PHA, Con A, or pokeweed. The failure to find an AA effect on MMR vaccine may simply be due to our failure to detect the effect of this vaccine or the AA effect may be antigen-specific. We do not have a specific explanation for the failure of MMR to stimulate PBMNC proliferation *in vitro* after immunization with the same vaccine *in vivo*; it may a defective vaccine or the way

of our testing. The lack of an AA effect on lymphocyte proliferation in response to polyclonal mitogens may be due to a relatively much higher stimulation by the mitogens compared to the stimulation caused by a specific antigen.

Our results showing no effect of dietary AA on proliferation of PBMNC cultured with mitogens are at variance to those results showing stimulation or inhibition of this response by the *in vitro* addition of AA to human lymphocytes and monocytes (36–38). In these studies AA concentrations up to 1.0 μg/mL enhanced the PHA-stimulated proliferation of human PBMNC cultured in lipid-poor medium (36); concentrations higher than 5 μg/mL inhibited their growth (37,38). The discrepancy between our results and those from previous studies may be due to the differences in the effects of free fatty acids (36–38) vs. those of the natural triglycerides (our study) and due to the differences in the concentrations and the metabolism of AA.

Previous studies have examined the effect of LA-rich diets on human IR, but not that of AA-rich diets. In a study with total dietary fat of 25 en%, increasing the LA content to 13 en% for 11 wk had no adverse effects on the IR of healthy men (1). Similar results with LA were obtained in a study with healthy women (2). LA is metabolized rapidly in humans, with only a limited conversion to AA, and thus should not have the same physiologic effects as the dietary AA. Direct comparisons between different studies should be made with caution, because several factors including total fat, chain length of different fatty acids and the ratios between them, antioxidant and other nutrient status, duration of feeding, and nutritional and health status of subjects determine the overall effects of diets on IR. Furthermore, not all indices of IR are affected equally by a given nutrient—as in the present study some indexes were stimulated (PBMNC proliferation with influenza vaccine), and others were unaffected by AA supplementation.

Infections of the upper respiratory tract were found in some individuals during the study; however such symptoms did not show any definite pattern and were randomly distributed in both dietary groups. At no time during the study did the basal (unstimulated) level of PBMNC proliferation for any given subject show more than two-fold variation. These observations indicate that the stimulation of IR was caused by AA and not by any latent viral infection. Furthermore these effects were not seen in the group devoid of AA, which also indicates that those were not caused by the stress of confinement at the metabolic unit. These observations indicate that the feeding of AA for a limited period did not significantly increase their risk for infections, although it may be increased in studies involving longer duration of AA feeding.

Even with the small number of subjects in the study (six in group 1; four in group 2), the results are encouraging, because there were no adverse effects of increased AA consumption as indicated in a previous study where esters of AA were fed (9). In our study, none of the indexes of IR tested were inhibited by AA feeding. Two of the indexes were significantly increased by AA, but the clinical significance of this increase is uncertain. It is possible that some other indexes would have been significantly affected, if AA was supplemented for more than 50 d or if there were more subjects in the study. These issues will be addressed in future studies with AA. Findings presented in this paper show neither any benefit from increased AA consumption by the healthy subjects nor any health risk if increased AA consumption was needed in cases of essential fatty acid deficiency. Further studies are needed before recommendations regarding daily intake of AA can be made.

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ARTICLE

Age-Related Changes in Catalytic Activity, Enzyme Mass, mRNA, and Subcellular Distribution of Hepatic Neutral Cholesterol Ester Hydrolase in Female Rats

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ABSTRACT: Activity and protein mass of hepatic neutral cholesteryl ester hydrolase (CEH) were measured in liver cytosol and washed microsomes of female Sprague-Dawley rats aged 3, 4, 7, 9, 12, and 16 wk. CEH mRNA was also measured. The microsomal component varied with age and contributed a greater fraction of total activity in females than previously reported in males. Nevertheless, the cytosolic component accounted for 62–80% of activity and 77–94% of immunoreactive protein in postmitochondrial fractions. Cytosolic and microsomal CEH specific activities, relative to total protein, decreased 94 and 83%, respectively, from 3 to 4 wk, prior to onset of puberty at 5 wk, and increased 360 and 137%, respectively, from 12 to 16 wk. These results contrast with an earlier study, in which cytosolic CEH activity of males increased with puberty and declined after 12 wk. Although cytosolic CEH was activated by protein kinase A and inhibited by alkaline phosphatase treatment at all ages, protein kinase activation peaked at 4 wk, coinciding with the initial decrease in specific activity. Specific activity in cytosol and microsomes correlated with CEH mass at all ages, suggesting that this CEH accounts for most variation in cellular activity. In contrast, CEH mRNA varied little from 3–16 wk, indicating that transcriptional regulation does not make a major contribution to the variation in CEH activity and mass in females, although it may make an important contribution to male–female differences in CEH expression. Specific activities of cytosolic and microsomal CEH, relative to immunoreactive CEH protein mass, exhibited changes consistent with posttranslational regulation. These results indicate gender-specific multivalent regulation of hepatic CEH by posttranslational mechanisms during development of female rats.
Lipids 32, 463–470 (1997).

Hepatic cholesterol synthesis, degradation, and excretion undergo extensive changes during aging (1–6). The hepatic levels of free and esterified cholesterol are regulated by the combined activities of 3-hydroxy-3-methylglutaryl–CoA (HMG–CoA) reductase, cholesterol 7α-hydroxylase (C7αH), acyl-CoA:cholesterol acyl transferase (ACAT), and cholesterol ester hydrolase (CEH). Although age-related changes in HMG–CoA reductase, C7αH, and ACAT are reported in the literature (2,4,6,7–13), these studies did not address the effects of aging on CEH. Other studies have provided evidence that the rat hepatic neutral cytosolic CEH is highly regulated in concert with the other three enzymes (14–16). Moreover, reported activation of CEH by protein kinase is consistent with a role for this enzyme in regulation of cholesterol homeostasis (17). Erickson *et al.* (18) recently reported very low levels of cytosolic CEH activity during postpartum development of male and female rats. However, activity was not measured under optimal conditions for cytosolic CEH. Moreover, enzyme protein levels were not reported in that study.

Although sex-related differences in cholesterol metabolism have been reported in experimental animals and humans (19–24), most of the available data result from studies on HMG–CoA reductase, the rate-limiting enzyme in cholesterol synthesis, and C7αH, the rate-limiting enzyme in bile acid synthesis. Choi *et al.* (25) reported that the activities of HMG–CoA reductase and C7αH are higher in female than in male rats and that the regulatory mechanisms for these enzymes are more sensitive in female than in male rats. Carlson *et al.* (21) also reported higher activities for these enzymes in female rats and that these differences became apparent at the time of sexual maturity. In contrast, Innis (24) reported higher levels of ACAT activity in young male rats than in females of the same age. However, sex-related differences in cytosolic CEH have yet to be reported.

The major cytosolic cholesterol ester hydrolyzing enzyme in rat liver has been purified and characterized in this laboratory (17,26–28) and its cDNA identified, cloned, and expressed (29). These studies indicated that CEH is predominantly cytosolic in male rats, although a minor fraction of activity and immunoreactive protein persisted in washed

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; C7αH, cholesterol 7α-hydroxylase; cAMP, adenosine 3′,5′-cyclic monophosphate; CEH, cholesterol ester hydrolase; HMG–CoA, 3-hydroxy-3-methylglutaryl–CoA; PVDF, polyvinylidene difluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis, SEM, standard error of the mean; SSC, 3.0 M sodium chloride and 0.3 M sodium citrate, pH 7.0; TCA, trichloroacetic acid.

microsomes (27). The properties which differentiate this enzyme from other esterases and lipases are reported and discussed elsewhere (28,29). We recently reported age-related changes in mRNA, protein mass, and specific activity of cytosolic CEH in male rats aged 6–24 wk, before and after puberty (30). In the current study we have measured hepatic neutral cytosolic, and microsomal CEH specific activities, cytosolic and microsomal CEH masses, and CEH mRNA levels in female rats, aged 3–16 wk, before and after puberty, in order to determine the effects of sex, puberty and age on CEH, identify potential regulatory mechanisms, and elucidate the role of this enzyme in cholesterol homeostasis during postpartum development.

MATERIALS AND METHODS

Chemicals and supplies. Cholesterol [1-^{14}C] oleate (56.6) mCi/mmol) was purchased from New England Nuclear (Boston, MA). All solvents were purchased from Fisher Scientific (Columbia, MD). ATP disodium salt, $MgCl_2$, adenosine 3′,5′-cyclic monophosphate (sodium salt), and alkaline phosphatase (Type IX from bovine liver) were purchased from Sigma Chemical Co. (St. Louis, MO). GENESCREEN membrane was purchased from NEN Research Products (Boston, MA). SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot supplies were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of analytical grade.

Sprague-Dawley rats with certified dates of birth were purchased from Zivic Miller Laboratories (Zellenople, PA) and fed TEKLAD Rodent Diet (Harlan Teklad, Madison, WI) *ad libitum* until use. Rats were maintained on a uniform 12-h light/12-h dark cycle at all times.

Preparation of cytosol. Rats were euthanized by decapitation and livers were removed and washed in 20 mM Tris HCl buffer, pH 7.5, containing 5 mM 2-mercaptoethanol, 100 mM sucrose and 80 mM KCl. Livers were homogenized with a loose Teflon pestle in 2 mL buffer/g tissue buffer. Cytosol (104,000 × *g* supernatant, S104) was prepared by differential centrifugation as described elsewhere (17). The pellet was resuspended in the same volume of homogenizing buffer and centrifuged again at 104,000 × *g* to obtain washed microsomes. This procedure has been used to remove loosely associated CEH protein and activity from male rat microsomes (27).

Enzyme assays. CEH activity was assayed in S104 and in microsomes by the radiometric method described previously (17). Cholesteryl oleate substrate was added as a droplet dispersion, which yields optimal levels of activity with the cytosolic CEH and enhances microsomal activity several orders of magnitude over phospholipid dispersions. Endogenous cAMP-dependent protein kinase was activated as described elsewhere (17), by preincubating the reaction mixture for 1–2 min with 1 mM $MgCl_2$, 5 mM ATP and 100 μM cAMP, followed by addition of substrate and an additional 30-min incubation. For alkaline phosphatase inactivation, the reaction mixture was preincubated with 0.1 unit phosphatase for 30 min, followed by addition of substrate and an additional 30-min incubation, as described previously (17). All assays were done in triplicate with two different protein concentrations within the linear range of the assay, for each of three rat livers/age group. All data were analyzed by the Student's *t*-test, analysis of variance and Bonferroni's test, with $P < 0.05$ as the minimal threshold for statistical significance.

Western blot analysis. Cytosolic or microsomal proteins were separated by SDS-PAGE and electroblotted onto Immobilon-polyvinylidene difluoride (PVDF) membranes using the Bio-Rad Trans Blot Cell. Western blots were reacted with polyclonal antibody to hepatic cytosolic CEH and developed as described previously (27), substituting 2% gelatin in TNT (20 mM Tris containing 500 mM NaCl and 0.05% Tween 20, pH 8.0) for 5% nonfat dry milk in the blocking solution. For quantitation, Western blots were scanned at 350 nm with a Shimadzu densitometer (Tokyo, Japan). A standard curve was constructed using different concentrations of hepatic CEH purified to a single band on SDS-PAGE by the procedure of Ghosh and Grogan (26). The standard curve was linear over the range of CEH protein concentrations measured in rat liver cytosol ($R = 0.97$). Three different concentrations of cytosolic protein were analyzed from each of three rat livers. Each of these concentrations yielded CEH values in the linear range of the standard curve and equivalent values when normalized for total cytosolic protein.

Northern blot analysis. Total RNA was prepared from isolated livers using TriReagent according to the manufacturer's instructions. An aliquot estimated to contain 10 μg of total RNA was electrophoresed on a 1% agarose gel in the presence of formaldehyde. The RNA was stained with ethidium bromide and the integrity of the isolated RNA verified by presence of 28*S* and 18*S* rRNA bands. According to the manufacturer's instructions, RNA was transferred to GENESCREEN membrane and simultaneously hybridized to ^{32}P-labeled full-length cDNA (29) for CEH and ^{32}P-labeled 0.8 kb probe for cyclophilin mRNA, used as a constitutive internal loading standard (31). After a high stringency wash (0.2 × SSC + 0.1% SDS at 65°C for 15 min), positive hybridization was detected by exposure to Kodak-2 films (Eastman Kodak Co., Rochester, NY) for 18 h at −70°C. Autoradiograms were scanned with a densitometer for quantitation and values normalized to cyclophilin.

Protein estimation. Protein was trichloroacetic acid (TCA)-precipitated and estimated by the bicinchoninic acid procedure (32).

RESULTS

Specific activity, mass, and mRNA levels of hepatic cytosolic CEH were measured in female rats at 3, 4, 7, 9, 12, and 16 wk after birth. As seen in Figure 1A, CEH specific activity (−protein kinase) decreased 94% from 3 wk to 4 wk of age ($P < 0.001$). It remained at this level through 12 wk and then in-

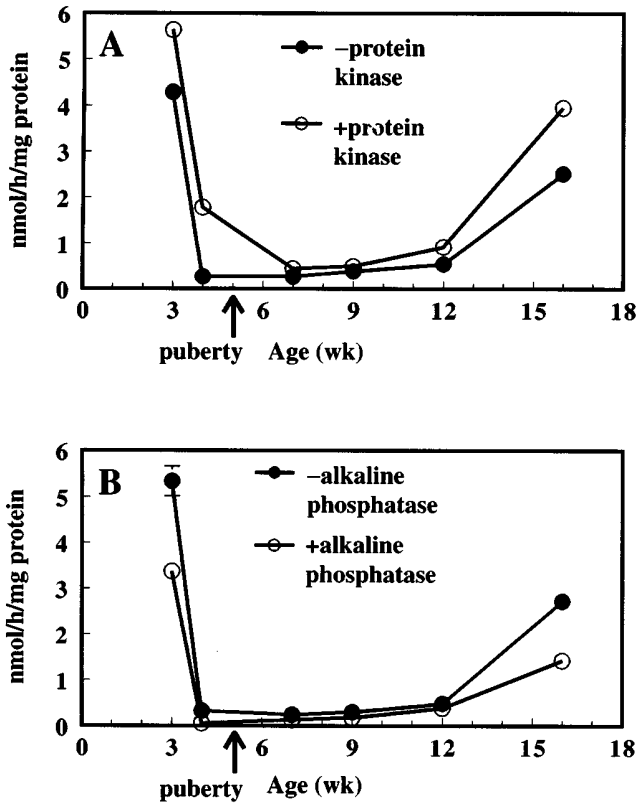

FIG. 1. Hepatic cytosolic CEH activity in female rats of various ages, with and without protein kinase A (Fig. 1A) or alkaline phosphatase (Fig. 1B) treatments. Figure 1A: CEH activity was measured radiometrically in 104,000 × *g* supernatants, in presence and absence of protein kinase A cofactors (1 mM $MgCl_2$, 5 mM ATP, 100 μM cAMP) added 1–2 min prior to addition of cholesteryl oleate substrate. Figure 1B: CEH was measured in 104,000 × *g* supernatants after incubation with or without alkaline phosphatase for 30 min. Arrow indicates onset of puberty. Each data point is the mean ± SEM for 3–6 rats, each rat was assayed in triplicate using two protein concentrations. Where not visible, error bars are within symbols.

creased again. By 16 wk, CEH specific activity had increased by 360% over the level in 12-wk-old rats ($P < 0.001$).

Previous studies with adult male rats have shown 100–140% stimulation of hepatic cytosolic CEH by cAMP (17), apparently mediated by endogenous cAMP-dependent protein kinase (30,33). These earlier studies also showed that this CEH is inactivated by exogenous alkaline phosphatase as well as by an endogenous Mg^{+2}-dependent phosphatase in rat liver cytosol (17). To evaluate the contribution of protein phosphorylation to the observed changes in hepatic CEH activity in the female rat between 3 and 16 wk of age, CEH activity was assayed, in the presence or absence of cofactors for cAMP-dependent protein kinase, or in the presence or absence of alkaline phosphatase (Figs. 1,2). As shown in Figure 1A, activators of endogenous protein kinase A increased CEH activity in all age groups, although this activation did not substantially alter the relative activities among the various age groups. The magnitude of this activation relative to basal CEH activities (Fig. 2) did not differ significantly with age, except at 4 wk, when activation was 588%, 8–20-fold greater than that seen at other ages. This is consistent with the earlier report of a peak in endogenous protein kinase A activity in rats of this age (34) and suggests that the level of phosphorylation may play a more important role in regulation of CEH activity at this stage of development. As depicted in Figure 1B, treatment with alkaline phosphatase decreased CEH activity by 20–62%, consistent with previous reports of regulation by phosphorylation/dephosphorylation in males (17,30). However, the relative activities before and after phosphatase treatment remained substantially the same among the various age groups. Thus, neither protein kinase activation nor alkaline phosphatase treatment significantly altered the pattern of age-dependent changes in CEH activity, as seen in Figure 1.

In previous studies from this and other laboratories, the predominant hepatic neutral CEH activity in male rats was

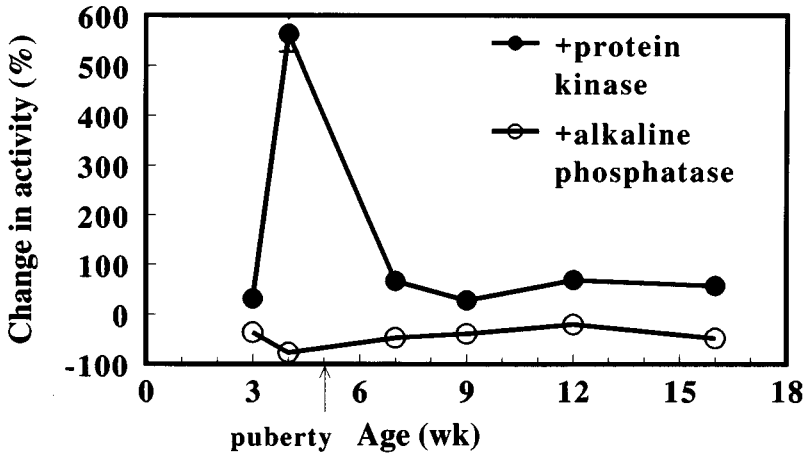

FIG. 2. Relative effects of protein kinase A and alkaline phosphatase on activity of hepatic cytosolic CEH in female rats of various ages. See legend, Figure 1, for treatments and assay conditions. Values are percentage difference from corresponding controls ± SEM for 3–6 rats/group, each rat assayed in triplicate, using two protein concentrations. Where not visible, error bars are within symbols.

isolated in the cytosolic fraction (26,35). In an earlier study from this laboratory, hepatic CEH activity and immunoreactive protein levels were very low (<10% of total activity) in microsomes from male rat liver (27). However, in the current study of female rats, microsomal CEH specific activities relative to total protein were similar to those of cytosol and much higher than those previously reported in males (Fig. 3A). Immunoreactive CEH mass (see Fig. 4 for representative Western blot) followed roughly the same general trend as specific activity in both cytosol and microsomes, decreasing from 3 to 7 wk and increasing thereafter (Fig. 3B), consistent with the view that this enzyme accounts for most of the observed variation in CEH activity in both subcellular fractions.

For comparison of the contributions of cytosolic and microsomal neutral CEH to the total cellular activity, the distribution of total neutral CEH catalytic activity and mass between the two subcellular fractions is shown in Table 1. Whereas the contribution of microsomal CEH activity varied from 20–38%, the contribution of microsomal immunoreactive protein varied from 6–23%. Although the distributions of mass and activity were similar only at 3 and 16 wk, both were consistently higher in cytosol than in microsomes at all ages. These data indicate that the predominant CEH activity is cytosolic in females, as previously reported in males (27), although the microsomal component contributes a substantially greater fraction of total activity in females. These data also reflect substantial differences in specific activity relative to CEH mass (nmol/h/μg CEH protein) between cytosolic and microsomal fractions at ages 4–12 wk, contrasting with specific activities at 3 and 16 wk, which were similar in the two fractions (see Table 1). This may reflect differential regulation of CEH in the two fractions.

Despite the variations with age and differences in specific activities relative to CEH mass between cytosol and microsomes, specific activities relative to cytosolic or microsomal protein correlated well (R = 0.94 and 0.96, respectively) with the corresponding specific masses of CEH (Fig. 5). However, as seen in Figure 5, regression lines for cytosol and microsomes diverged at low activities and masses and appeared to converge with higher activities and masses, consistent with

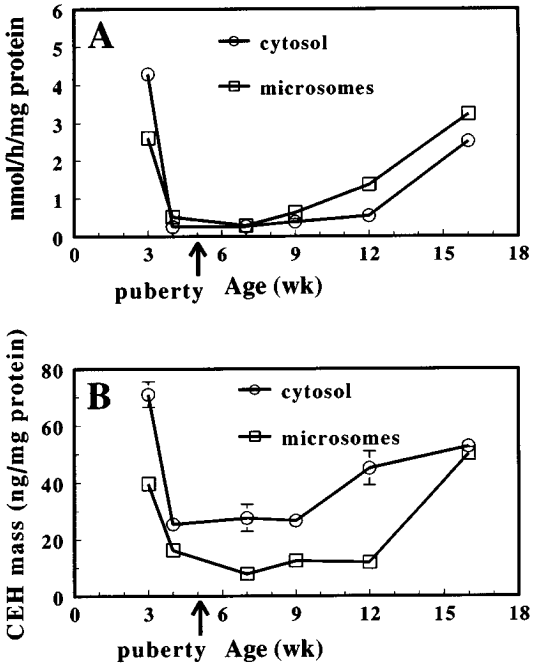

FIG. 3. Distribution of hepatic neutral CEH catalytic activity and mass between cytosol and microsomes from female rats of various ages. Figure 3A: CEH catalytic activity was measured in cytosol and resuspended washed microsomes as described in legend, Figure 1. Each data point is the mean ± SEM specific activity, relative to cytosolic or microsomal protein, for 3–6 rats, each rat assayed in triplicate, using two protein concentrations. Figure 3B: CEH mass was measured in cytosol or resuspended washed microsomes by quantitative Western blotting, as described in the Materials and Methods section. Each data point is the mean ± SEM CEH mass, relative to cytosolic or microsomal protein, for 3–6 rats; three different protein concentrations were assayed in the linear range of a standard curve obtained with an appropriate range of purified rat liver CEH. Where not visible, error bars are within symbols.

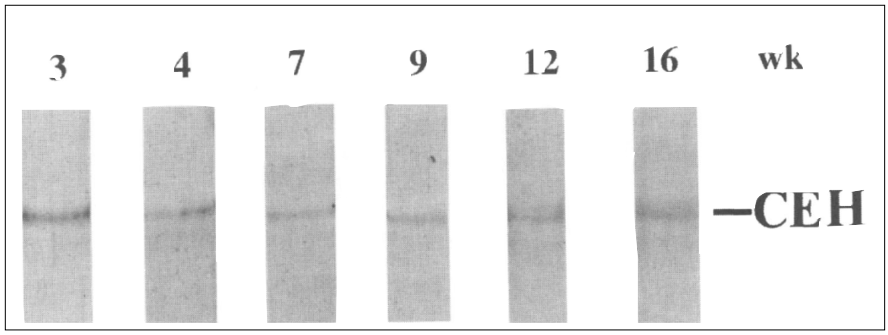

FIG. 4. Representative Western blot analysis of rat liver cytosols with polyclonal antibody to rat liver CEH, depicting variation of CEH mass in 3-, 4-, 7-, 9-, 12-, and 16-wk-old female rats. Blots were scanned densitometrically and values converted to corresponding masses by comparison with a standard curve obtained with varying concentrations of purified rat liver CEH. Resulting values are depicted in Figures 3B, 6, and 8B.

TABLE 1
Distribution of Neutral CEH Catalytic Activity and Anti-CEH Immunoreactive Protein Between Liver Microsomes and Cytosol in Female Rats of Various Ages

Age (wk)	Specific activity[a] (nmol/h/μg) CEH		Microsomal fractional content[b] (% total postmitochondrial)	
	Cytosol	Microsomes	CEH activity	CEH mass
3	62 ± 6	66 ± 2	24 ± 1	23 ± 2[k]
4	10 ± 1[c,h]	33 ± 5[d]	19 ± 2	8 ± 1[d,k]
7	9 ± 1[c,h]	36 ± 2[c,i]	22 ± 6	6 ± 1[d,j]
9	14 ± 2[d,h]	51 ± 6	36 ± 3[f,j]	13 ± 0[e]
12	13 ± 2[d,h]	122 ± 29[l]	38 ± 7	6 ± 1[d,j]
16	48 ± 1	66 ± 5	20 ± 2	15 ± 2[g]

[a]Specific activity relative to cholesterol ester hydrolase (CEH) mass was calculated as the ratio (specific activity relative to cytosolic or microsomal protein/specific CEH mass relative to cytosolic or microsomal protein). All values are means for 3–6 rats ± SEM.
[b]Microsomal fractional content for CEH activity = 100 (microsomal CEH activity/g liver)/[(microsomal CEH activity/g liver) + (cytosolic CEH activity/g liver)]; for CEH mass = 100 (microsomal CEH mass/g liver)/[(microsomal CEH mass/g liver) + (cytosolic CEH mass/g liver)]; statistical differences = [c]different from 3 wk, $P < 0.001$; [d]different from 3 wk, $P < 0.005$; [e]different from 3 wk, $P < 0.01$; [f]different from 3 wk, $P < 0.02$; [g]different from 3 wk, $P < 0.05$; [h]different from 16 wk, $P < 0.001$; [i]different from 16 wk, $P < 0.01$; [j]different from 16 wk, $P < 0.02$; [k]different from 16 wk, $P < 0.05$; [l]different from 7 wk, $P < 0.05$.

the previously cited convergence of specific activities (see Table 1). Inasmuch as the regression line for microsomal CEH passed through the origin, the immunoreactive CEH protein accounts for essentially all of the catalytic activity in this fraction and essentially all of the immunoreactive protein in this fraction appears to be active under the assay conditions. In contrast, the regression line for cytosolic CEH intercepts the mass axis at a protein mass > 0. Whereas this observation suggests that the immunoreactive protein accounts for the catalytic activity in cytosol, it also indicates that a substantial fraction of immunoreactive protein is not catalytically active, perhaps reflecting posttranslational regulation. Such a component of inactive CEH may account for the above-cited discrepancies in specific activities of cytosol and microsomes relative to CEH mass, which are most prominent at lower mass levels (see Table 1).

As shown in Figure 6, CEH mRNA levels measured by quantitative Northern blot analysis varied little, with the exception of a transient increase at 4 wk, which was not statistically significant ($N = 5$). Thus, transcriptional regulation does not appear to play an important role in the observed variation in CEH activity in females, although it may contribute to female/male differences.

DISCUSSION

This is the first reported measurement of CEH specific activity, mass, and mRNA in female rats and the first developmental study in which CEH specific activities are correlated with enzyme mass in both cytosolic and microsomal compartments. These results contrast markedly with those obtained in a similar study of developmental changes in CEH in male rats (30). In females, the specific activities relative to cytosolic protein dropped precipitously at 4 wk and remained at very low levels through 12 wk, after which it rose again (Fig. 1). In contrast, specific activities in male rats rose from 6 to 10 wk, and then declined after 12 wk (30). Thus, in females, highest specific activities were present just prior to onset of puberty (approximately 5 wk) and after 12 wk, whereas, in males, highest activities were seen just after onset of puberty (approximately 8 wk) and prior to 12 wk. These trends were also seen with CEH mass, which decreased in females with

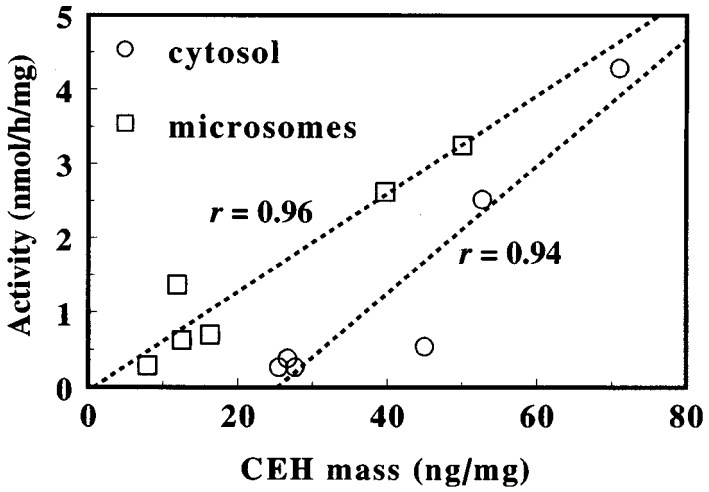

FIG. 5. Correlations between immunoreactive protein and catalytic activity in cytosolic and microsomal fractions of female rats of various ages. Broken lines depict best-fit linear regression for all cytosolic ($R = 0.94$) and microsomal ($R = 0.96$) values, respectively. Each data point is the mean for 3–6 rats. See Figure 4 for description of experimental procedures and standard errors of the means for both variables, which are contained within the symbols for most data points.

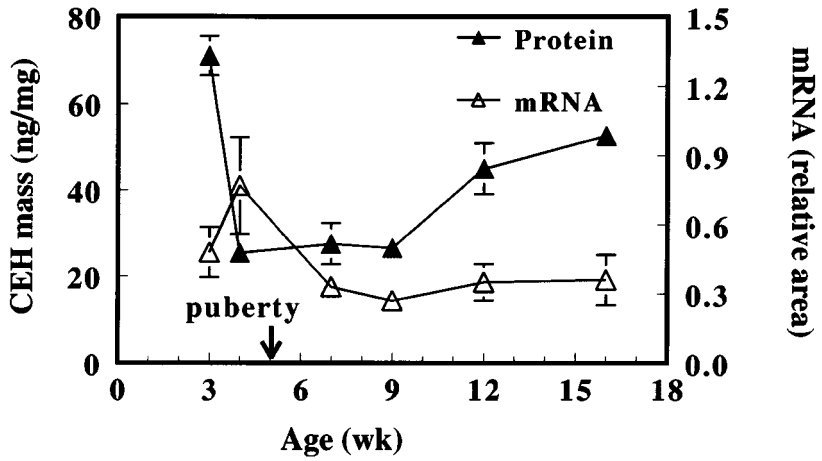

FIG. 6. Hepatic CEH mRNA and cytosolic CEH mass in female rats at various ages. CEH mRNA was measured (relative to constitutive cyclophilin mRNA) by quantitative Northern blot analysis with full length CEH cDNA (see Fig. 7, for representative autoradiogram), as detailed in the Materials and Methods section. CEH mass was measured by quantitative Western blot analysis as detailed in the Materials and Methods section. The arrow indicates the onset of puberty in female rats. Each data point is the mean ± SEM for 3–6 rats, each rat assayed in triplicate. Where not visible, error bars are contained within symbols.

onset of puberty and rose after 12 wk (Fig. 3), whereas it increased in males during puberty and declined after 12 wk (30). In contrast, whereas CEH mRNA levels in male rats corresponded to CEH activity and mass (30), mRNA levels in females did not parallel activity or mass (Fig. 6). Nevertheless, both CEH mass and mRNA levels were consistently lower in females (Fig. 6) than males (30) at comparable ages. These results suggest that age-related changes in CEH in female rats reflect primarily posttranscriptional mechanisms, whereas transcriptional regulation may contribute to the female/male differences in CEH activity and mass. The pattern of susceptibility to protein kinase A was also quite different in female rats (Fig. 2) in comparison with males, which showed a sustained increase in susceptibility after puberty (30).

Since marked changes in CEH mass, catalytic activity, and susceptibility to protein kinase A coincide with onset of puberty, this regulation may be mediated, directly or indirectly, by gonadal steroids. Consistent with this view are previous reports that hepatic CEH activity is suppressed by estradiol (36,37) and induced by progesterone (16,38). Other enzymes of cholesterol homeostasis are also differentially expressed in females and males or known to be regulated by gonadal steroid hormones. Whereas the activities of HMG–CoA reductase and C7H are reported to be greater in adult females than in males (25), ACAT activity was greater in males (24). Like CEH, HMG–CoA reductase and C7αH are reported to be stimulated by progesterone (38–40). Moreover, C7αH is also inhibited by estradiol (38). In contrast to CEH, ACAT is reported to be inhibited by progesterone (16,41,42) consistent with the antagonistic roles of these enzymes in the cholesteryl ester cycle.

Assignment of changes observed in females during weeks 3–4 to onset of puberty is confounded by the close proximity to weaning. Since female rats were weaned only 3 d prior to the initial 3-wk data points, it is possible that 3-wk data and/or changes occurring in the subsequent week may reflect alterations in diet or other maternal influences. Although no comparable data have been reported for female rats, similar studies with HMG–CoA reductase in male rats, which enter puberty 2 wk later than females, have shown transient increases in HMG–CoA reductase specific activity and mass after weaning (7,8). However, these changes occurred immediately and persisted for only a few days, after which specific activity and mass of HMG–CoA reductase returned to initial levels. Increases in HMG–CoA reductase and C7αH mRNA were also reported immediately after weaning (43), similar in magnitude to the increase in CEH mRNA at 4 wk in the current study (Fig. 6), although the duration of those changes was not reported. Nevertheless, the transient increase in susceptibility of CEH to activation by cAMP-dependent protein kinase at 4 wk (Fig. 2) is probably associated with onset of puberty, since it is absent at 3 wk, 3 d after weaning.

As suggested by earlier studies indicating that <10% of liver CEH activity or mass was present in adult male microsomes (27), these studies provide additional evidence that CEH is predominantly cytosolic, as initially proposed by Deykin and Goodman (35). Nevertheless, 6–23% of immunoreactive protein remained associated with washed microsomes and correlated well with catalytic activity (Table 1), indicating that this enzyme makes a major contribution to microsomal as well as cytosolic CEH activity.

Although microsomal CEH activity and mass generally paralleled cytosolic activity and mass (Fig. 3), the distribution of CEH mass between the subcellular fractions varied significantly with age (Table 1), suggesting that redistribution

may play a role in regulation of this enzyme. Moreover, the substantial variations in specific activity relative to CEH mass in both males and females among the various age groups (see Table 1) and between cytosolic and microsomal components suggest additional posttranslational mechanisms of regulation. One such mechanism is suggested by the variable susceptibility of this enzyme to modulation by phosphorylation/dephosphorylation, as indicated by this and previous studies cited earlier. An additional posttranslational mechanism is suggested by the earlier report of a cytosolic factor which exhibits variable inhibitory activity with age (44). Since this factor was more active in adult females than in males (44), variability in its expression could account for the greatly reduced specific activity of cytosolic CEH in females at 4, 7, 9, and 12 wk, in the absence of concomitant changes in microsomal specific activity (Table 1).

These studies suggest that the hepatic neutral CEH plays an important role in regulation of cholesterol homeostasis in the female rat during development and that its regulation is complex, multivalent, and distinct from the pattern expressed in males. Although it is not yet clear how differential expression may serve the needs of females and males at different stages of development, CEH is apparently regulated in concert with HMG–CoA reductase, C7αH and ACAT, to maintain adequate levels of intracellular free cholesterol for bile acid synthesis, biliary cholesterol secretion, and assembly of membranes and lipoproteins. As with HMG–CoA reductase, transcriptional regulation of CEH is complemented by other modes of regulation, to permit independent or opposing modulation of the two enzymes capable of increasing hepatic free cholesterol.

ACKNOWLEDGMENT

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Metal Ion Stimulation of Phospholipase D-Like Activity of Isolated Rat Intestinal Mitochondria

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ABSTRACT: Presence of phospholipase D-like (PLD) activity in the intestinal mitochondria was identified using endogenous phospholipids as substrate. The enzyme had a pH optimum of 6.5, did not show *trans*-phosphatidylation activity in the presence of ethanol or butanol, and the product formed was phosphatidic acid (PA). This was confirmed by separation of reaction products by high-performance liquid chromatography and analysis of composition of the PA formed which gave phosphate/fatty acid ratio of 1:2. PLD-like activity was further confirmed by the formation of ethanolamine and choline as products of enzyme action. This activity was stimulated by various metal ions; when stimulated by Mg^{2+} and Ba^{2+}, it hydrolyzed both phosphatidylcholine and phosphatidylethanolamine, and when stimulated by Ca^{2+}, it preferentially hydrolyzed phosphatidylethanolamine. There was no requirement for sodium oleate for the PLD-like activity in mitochondria. These results suggest that intestinal mitochondria have an active PLD-like enzyme which differs in certain properties from phospholipase D from other tissues.

Lipids 32, 471–479 (1997).

A number of studies have shown that phosphatidic acid (PA) is involved in cell signaling (1,2). PA can either be formed by the action of phospholipase D (PLD) on phospholipids or by phosphorylation of diacylglycerol. PLD can be activated in response to various stimuli and phorbol esters activate PLD through protein kinase C (3,4). Protein kinase C activation of PLD takes place by both phosphorylation-dependent and independent mechanisms (5,6). Lipopolysaccharide stimulates cellular PLD activity, and this requires an increase in intracellular Ca^{2+} (7). Exposure to oxygen-derived free radicals also stimulates cellular PLD activity (8,9). Using PLD from various sources, it has been shown that divalent metal ions can activate this enzyme (10,11).

Calcium plays an important role in many cellular functions, and intracellular calcium is stored mainly in endoplasmic reticulum and to a small extent in the mitochondria (12). Under certain pathological conditions, such as oxidative stress, increase in cytosolic Ca^{2+} occurs and this in turn increases the Ca^{2+} sequestration by mitochondria (13,14). It has been proposed that PA may act as a Ca^{2+} ionophore (15,16) and facilitate the influx of Ca^{2+} through the plasma membrane. Although presence of PLD activity has been shown in liver, skeletal muscle and brain mitochondria, very little is known on the properties of mitochondrial enzyme (17–19). Since mitochondria are important in calcium homeostasis and PA might play a role in this process, the present study looks at the possible presence of PLD-like activity and its properties in the intestinal mitochondria.

MATERIALS AND METHODS

Various lipid standards, Hepes, fluorescamine, *p*-bromophenacylbromide (pBPB), chlorpromazine (CHLP), and bovine serum albumin (BSA) were all obtained from Sigma Chemical Company (St. Louis, MO). Standard phosphatidylethanol and phosphatidylbutanol were prepared using egg phosphatidylcholine and cabbage PLD in presence of ethanol or butanol (20). All other chemicals used were of analytical grade.

Preparation of mitochondria. Rats weighing 150–200 g, fasted overnight, were decapitated, the small intestine removed and washed with ice-cold saline. Mitochondria were isolated from enterocytes as described by Masola and Evered (21). Isolated mitochondrial fraction was suspended in EGTA-free medium containing 250 mM sucrose/5 mM Hepes pH 7.4 and stored in ice at protein concentration of 8–10 mg/mL. Isolated mitochondria were relatively pure as judged by the mitochondrial marker enzyme succinic dehydrogenase. Microsomal and lysosomal contamination in the mitochondrial preparation were checked by assaying marker enzymes, namely, glucose-6-phosphatase, acid phosphatase and arylsulfatase, and these activities were not detectable. Protein was measured using BSA as standard (22).

Enzyme assay. Mitochondrial PLD-like activity was measured using endogenous mitochondrial phospholipids as substrate. Mitochondria (1 mg protein per mL in 0.25 M sucrose, 5 mM Hepes buffer pH 7.4) were incubated at 37°C for 30 min. Following incubation, total lipids were extracted by

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Abbreviations: PA, phosphatidic acid; pBPB, *p*-bromophenacylbromide; PLD, phospholipase D; DAG, diacylglycerol; CHLP, chlorpromazine; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

Bligh and Dyer's method (23) and the PA was separated by thin-layer chromatography (TLC) and quantitated by phosphate estimation. Extracted lipids were spotted on silica gel G plates impregnated with 0.5 M oxalic acid and separated using the solvent system chloroform/methanol/conc. HCl (85:13:0.5, by vol) (24). PA spots corresponding to standard were identified by iodine exposure, scraped, and eluted from the plates. PA was quantitated by phosphate estimation after acid digestion (25). PA formed was also separated and identified along with standard using two other solvent systems (26,27). Formation of PA was further confirmed by separation of extracted lipids on TLC followed by isolated PA spot separated by high-performance liquid chromatography (HPLC) (28). A normal-phase silica 5-µm column was used with the solvent gradient of 1–9% water in hexane/2-propanol (3:4, vol/vol) at a flow rate of 1 mL/min and the eluents monitored at 220 nm. Transphosphatidylation was checked by inclusion of 300 mM ethanol or butanol in the incubation mixture, and extracted lipids were separated on silica gel G plates using the upper phase of ethyl acetate/isooctane/acetic acid/water (13:2:3:10, by vol) (27). PA formation in the presence of ethanol was further checked by HPLC. Metal ion stimulation of phosphatidylcholine degradation generated choline which was separated by TLC, using the solvent system methanol/0.6% NaCl/ammonia (50:50:5, by vol). This was identified after exposure to iodine vapor (29).

Lipid analysis. Neutral lipids were separated on silica gel G plates using the solvent system hexane/diethyl ether/acetic acid (80:20:1, by vol). Spots were identified by iodine exposure, scraped, and eluted. Cholesterol (30), diglycerides, and triglycerides (31) were estimated as described. Free fatty acids were methylated and quantitated by gas chromatography after separation on a 5% EGSS-X column. Heptadecanoic acid was used as internal standard. Individual phospholipids were separated on silica gel H plates using the solvent system chloro-form/methanol/acetic acid/water (25:15:4:2, by vol) (32) and quantitated by phosphate estimation after acid hydrolysis. Individual aminophospholipids were also quantitated after derivatization with fluorescamine and separation on silica gel H plates impregnated with 3% magnesium acetate using the solvent system chloroform/methanol/NH_4OH/water (60:40:5:2, by vol) (33). Eluted individual spots were quantitated using Shimadzu SF 5000 spectrofluorometer (Tokyo, Japan) with excitation at 395 nm and emission at 468 nm.

Separation and analysis of ethanolamine. Following incubation, enzyme activity was terminated with 10% trichloroacetic acid. After cooling on ice, the mixture was transferred

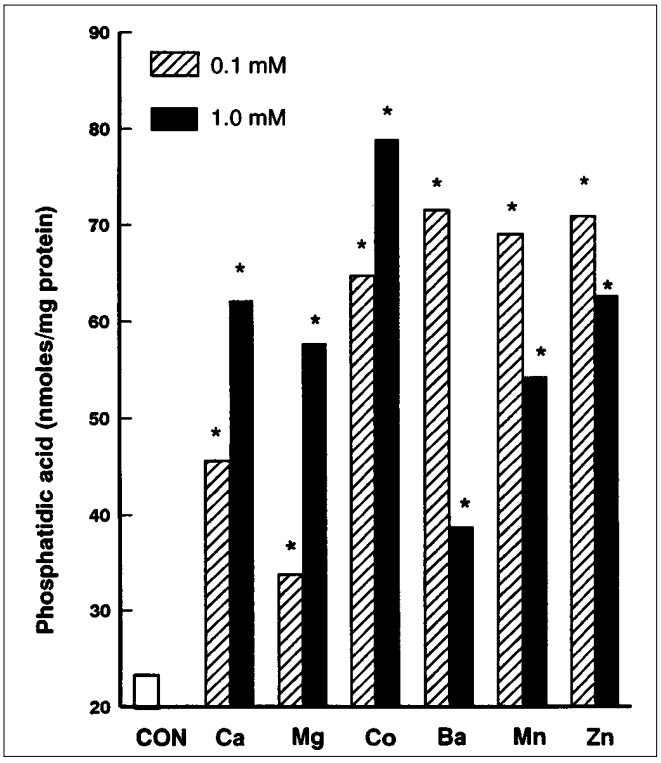


FIG. 1. Effect of various metal ions (at 0.1 and 1 mM final concentration) on phospholipase D activity of intestinal mitochondria. Each value represents mean ± SEM of three separate estimations. *$P < 0.05$ versus control (CON) incubated mitochondria.

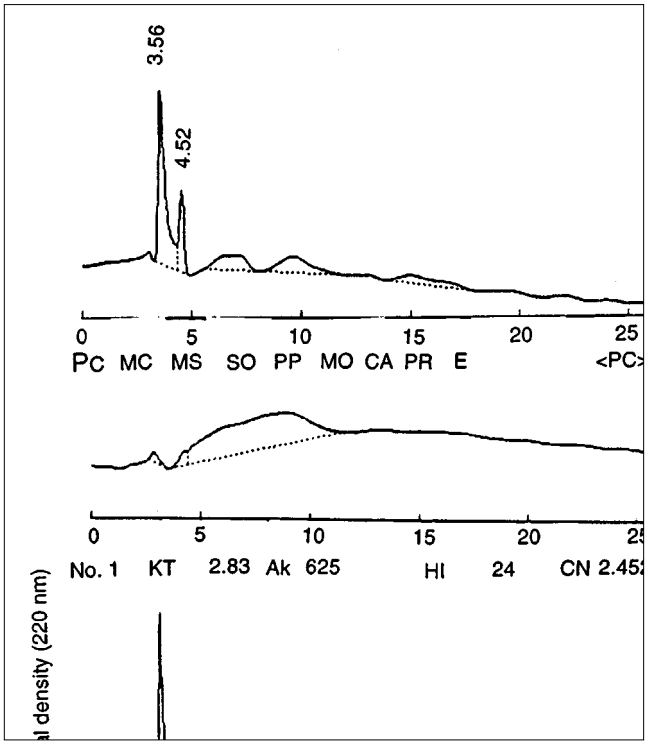


FIG. 2. Thin-layer chromatographic separation of phosphatidic acid (PA) and phosphatidylethanol. Silica gel G plate was developed using the upper phase of the solvent system ethylacetate/isooctane/acetic acid/water (13:2:3:10, by vol). Lane 1 contains PA and phosphatidylethanol standard. Lane 2 represents Ca^{2+}-activated mitochondrial PA formation in the absence of ethanol. Lane 3 represents PA formation by Ca^{2+} activation in presence of 200 mM ethanol.

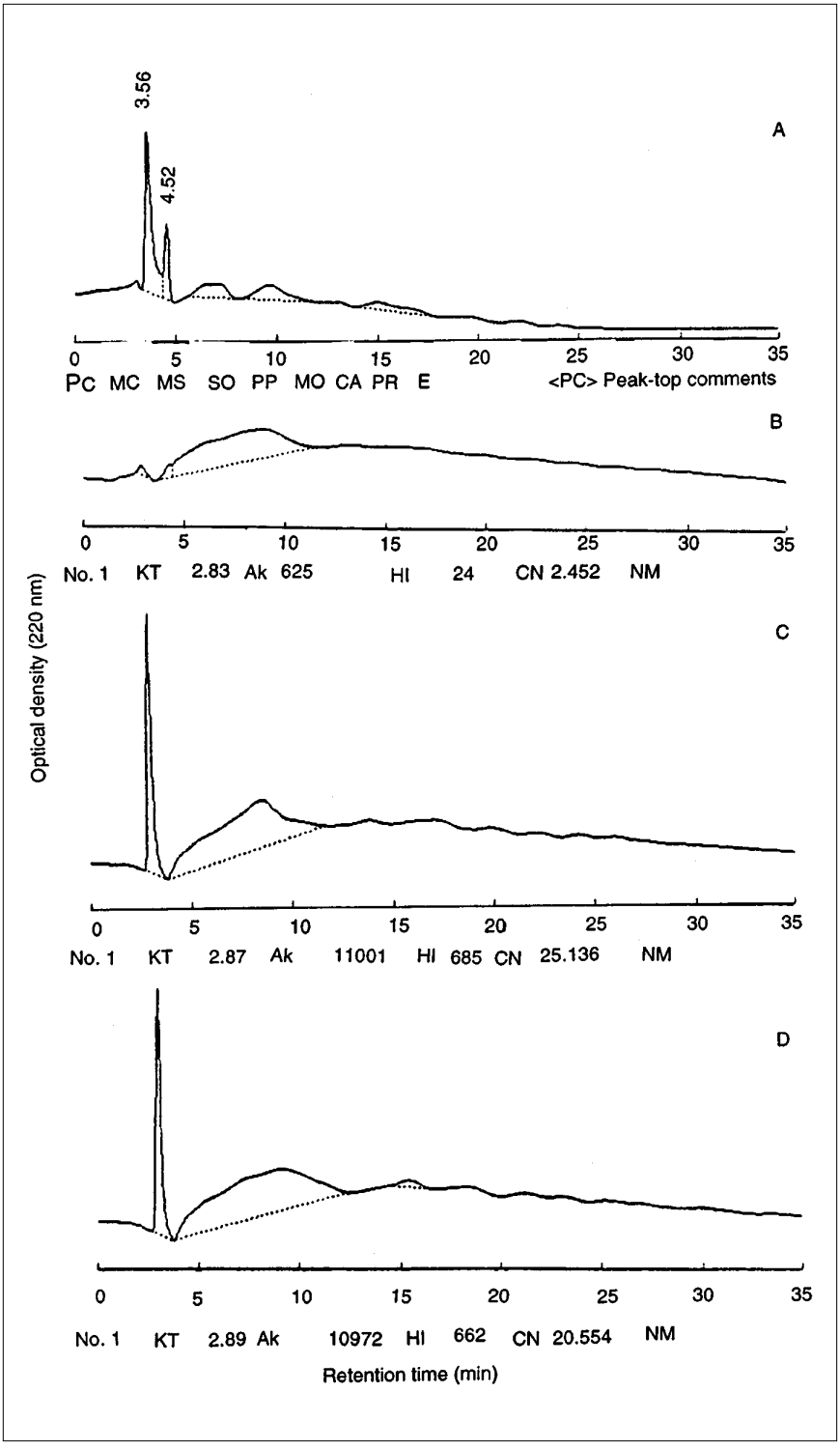


FIG. 3. High-performance liquid chromatography separation of phosphatidic acid formed in mitochondria; (A) standard phosphatidic acid and phosphatidylethanol; (B) unincubated mitochondria; (C) mitochondria incubated with 1 mM Ca^{2+}; (D) mitochondria incubated with 1 mM Ca^{2+} + 200 mM ethanol. Total lipids were extracted. Phosphatidic acid separated by thin-layer chromatography was eluted and run on high-performance liquid chromatography as described in the text.

TABLE 1
Composition of Isolated Phosphatidic Acid[a]

	C_o	C_{30}	$+Ca^{2+}$
Phosphate (nmole)	10.50	20.98	60.70
Fatty acids (nmole)			
16:0	10.00	18.75	57.45
18:0 and 18:1	5.70	10.10	28.45
18:2	6.26	8.45	15.20
20:4	3.10	5.40	19.00
Total fatty acid	25.56	42.70	120.05
Ratio of phosphate to fatty acid	1:2.24	1:2.03	1:1.97

[a]C_o: Control unincubated mitochondria; C_{30}: mitochondria incubated for 30 min without any activator. For calcium activation, mitochondria were incubated with 1 mM Ca^{2+} for 30 min.

to a test tube, shaken on a vortex mixer for a minute, left for 15 min and then centrifuged. The sediment was washed twice with 2 mL of 3% trichloroacetic acid and the supernatants pooled together. Trichloroacetic acid from the supernatant was removed by repeated extraction with diethyl ether, and the solution was dried with nitrogen. The samples were dissolved in 150 µL of 0.2 M boric acid pH 9.0, and 50 µL of fluorescamine (2 mg in 0.25 mL acetone) was added. The reaction with fluorescamine was allowed to proceed for at least 5 min to hydrolyze the excess reagent. After derivatization, the samples were separated by TLC on silica gel G plates (solvent system, chloroform/methanol/conc. ammonia/water (60:40:5:2.5, by vol). Fluorescamine-labeled ethanolamine was seen under ultraviolet light. This was scraped and eluted with 3.5 mL of methanol/4% ammonia (95:5, vol/vol) and quantitated using a spectrofluorometer with an excitation at 395 nm and emission at 468 nm. Standard ethanolamine was also derivatized with fluorescamine, and the fluorescence response of standard derivatized ethanolamine was linear in the concentration range of 25 to 1000 µM.

Statistical analysis. The values represent mean ± SEM of three separate estimations, and Mann-Whitney U-test was done to compare the changes.

RESULTS

The PLD-like activity in the isolated intestinal mitochondria was determined by measuring the PA formation from endogenous mitochondrial phospholipids. Figure 1 shows the effect of various divalent metal ions (Ca^{2+}, Mg^{2+}, Ba^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+}) at two different concentrations (0.1 and 1 mM) on PA formation. As shown, Ca^{2+}, Mg^{2+} and Co^{2+} activated at both concentrations, whereas with Ba^{2+}, Mn^{2+} and Zn^{2+}, maximum activation was seen at 0.1 mM concentration. Mitochondrial PLD-like enzyme did not show transphosphatidylation activity. Inclusion of either 200 and 300 mM ethanol or butanol in the incubation medium did not result in the formation of phosphatidylethanol or phosphatidylbutanol when stimulated by metal ions. Under these conditions, all the product formed was recovered as PA (60.7 ± 1.5 nmoles PA in presence of Ca^{2+} + ethanol, 61.8 ± 2.4 in presence of Ca^{2+} alone, 55.4 ± 1.7 in presence of Mg^{2+} + ethanol and 53.9 ± 1.9 in presence of Mg^{2+} alone). Figure 2 shows the TLC separation of PA and phosphatidylethanol after Ca^{2+} activation of mitochondrial PLD-like activity which showed the absence of phosphatidylethanol formation in the presence of ethanol. This was further confirmed by HPLC separation of PA formed in the absence and presence of ethanol. Control mitochondria had very little PA which was increased when incubated with Ca^{2+}. In the presence of ethanol and Ca^{2+}, all the product was recovered as PA, as evidenced by HPLC separation (Fig. 3). Table 1 shows the phosphate content and fatty acid composition of PA isolated

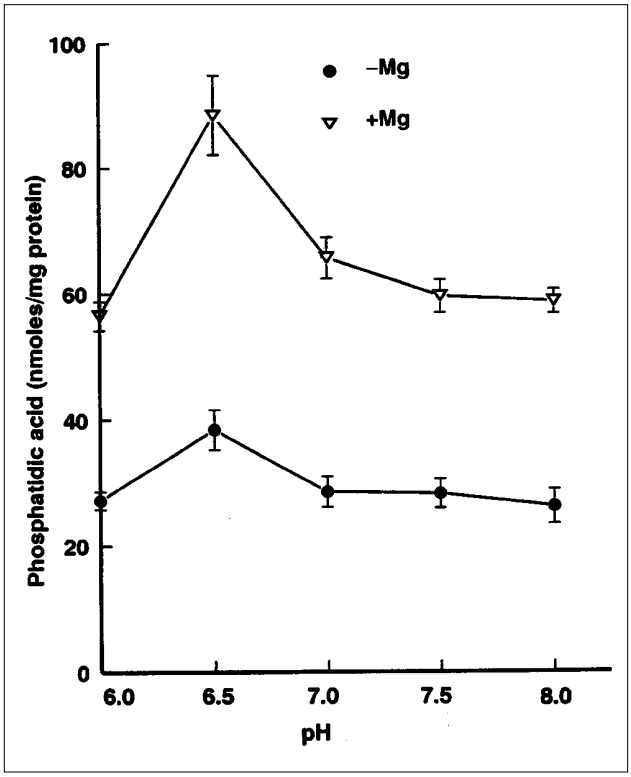


FIG. 4. Effect of different pH on phospholipase D activity of intestinal mitochondria in the presence and absence of 1 mM Mg^{2+}. Each value represents mean ± SEM of three separate estimations.

TABLE 2
Neutral Lipid Composition (nmole/mg protein) of Intestinal Mitochondria Incubated with Metal Ions[a]

Lipids	Control	Control incubated	Incubated in presence of Ca^{2+}	Mg^{2+}	Ba^{2+}
Total cholesterol	139 ± 11.2	142 ± 9.9	133 ± 7.4	136 ± 12.4	141 ± 2.5
Triglyceride	94 ± 6.6	86 ± 5.3	93 ± 5.3	94 ± 2.3	88 ± 4.1
Diglyceride	59 ± 4.5	59 ± 3.6	56 ± 4.7	59 ± 6.2	58 ± 5.1
Free fatty acids	101 ± 12.0	116 ± 9.7	114 ± 10.5	112 ± 14.9	113 ± 2.8

[a]Each value represents mean + SEM of three separate estimations. Ca^{2+} (1 mM), Mg^{2+} (1 mM), and Ba^{2+} (0.1 mM) (final concentration) were used.

from the reaction mixture, and the ratio of phosphate to fatty acid was found to be 1:2, which confirms that the product formed was indeed PA. To rule out the possibility of PA formation from diacylglycerol (DAG) by DAG kinase, isolated mitochondria were incubated with 400 μM DAG (exogenous) and 1 mM ATP at 37°C for 30 min with and without metal ions. Following this, lipids were extracted; DAG and PA were quantitated. The addition of exogenous DAG did not alter the PA formation in the presence of metal ions (Ca^{2+} alone: 59.8 ± 2; Ca^{2+} + DAG: 61 ± 2.6; Mg^{2+} alone: 54 ± 1.9; and Mg^{2+} + DAG: 55 ± 2.3 nmoles/mg protein). Levels of DAG also remained the same in control and metal ion-treated samples (data not shown). Mitochondrial PLD-like enzyme showed a pH optimum of 6.5, and this remained the same even when activated by metal ion (Fig. 4). The possible influence of endogenous metal ions on mitochondrial PLD activity was checked in the absence and presence of 2, 5, and 10 mM EDTA. Unincubated mitochondria had a PA content of 12.58 ± 1.36 nmoles/mg protein, which increased to 23.06 ± 3.16 nmoles after incubation for 30 min without any metal ions. Inclusion of various concentrations of EDTA did not alter the PA content (data not shown).

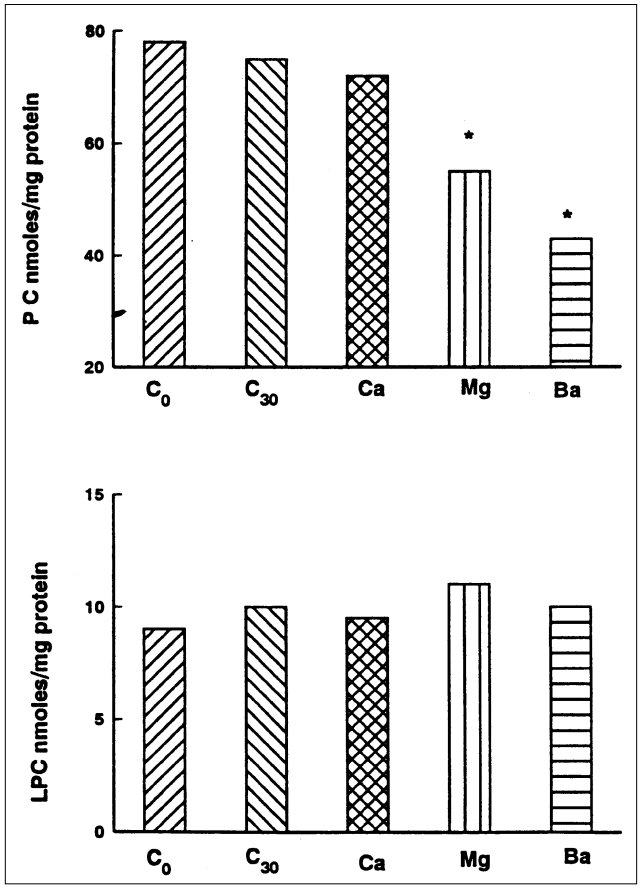


FIG. 5. Phosphatidylcholine (PC) and lyso PC (LPC) content of mitochondria incubated with Ca^{2+} (1 mM), Mg^{2+} (1 mM), and Ba^{2+} (0.1 mM). C_o represents unincubated mitochondria, and C_{30} represents mitochondria incubated for 30 min without metal ions.

PLD is known to act on both phosphatidylcholine (PC) and phosphatidylethanolamine (PE). To check the endogenous substrate for mitochondrial enzyme, mitochondrial lipids were analyzed after metal ion activation of PLD-like activity. As can be seen from Table 2, no significant change was seen in neutral lipids. Phospholipid separation by TLC indicated that in the case of Ca^{2+} activation, PLD-like enzyme preferentially used PE as substrate, whereas with Mg^{2+} and Ba^{2+}, both PC and PE were hydrolyzed. Figure 5(A and B) shows PC and lyso PC content of mitochondria after metal ion activation of the enzyme. As can be seen, with both Mg^{2+} and Ba^{2+} stimulation, PC content decreased whereas Ca^{2+} stimulation did not alter the PC content. Moreover there was no significant change in the content of lyso PC after metal-ion activation. PC hydrolysis by PLD-like enzyme in the presence of Mg^{2+} and Ba^{2+} was further confirmed by the formation of choline which was separated and identified by TLC and is shown in Figure 6. Ca^{2+} stimulation of the enzyme did not produce choline, whereas Mg^{2+} and Ba^{2+} stimulation resulted in the formation of choline; and the maximum amount of choline formation occurred with Ba^{2+} stimulation of enzyme. Amino phospholipids were quantitated by fluorescamine derivatization followed by TLC separation. As shown in Figure 7(A and B), Ca^{2+}, Mg^{2+}, or Ba^{2+} stimulation decreased the PE content, and there was no significant change in the level of lyso PE following metal ion activation. Ethanolamine, the other product of PLD degradation of PE, was separated by TLC after fluorescamine derivatization and is shown in Figure 8. Quantitation of ethanolamine formed showed that all three

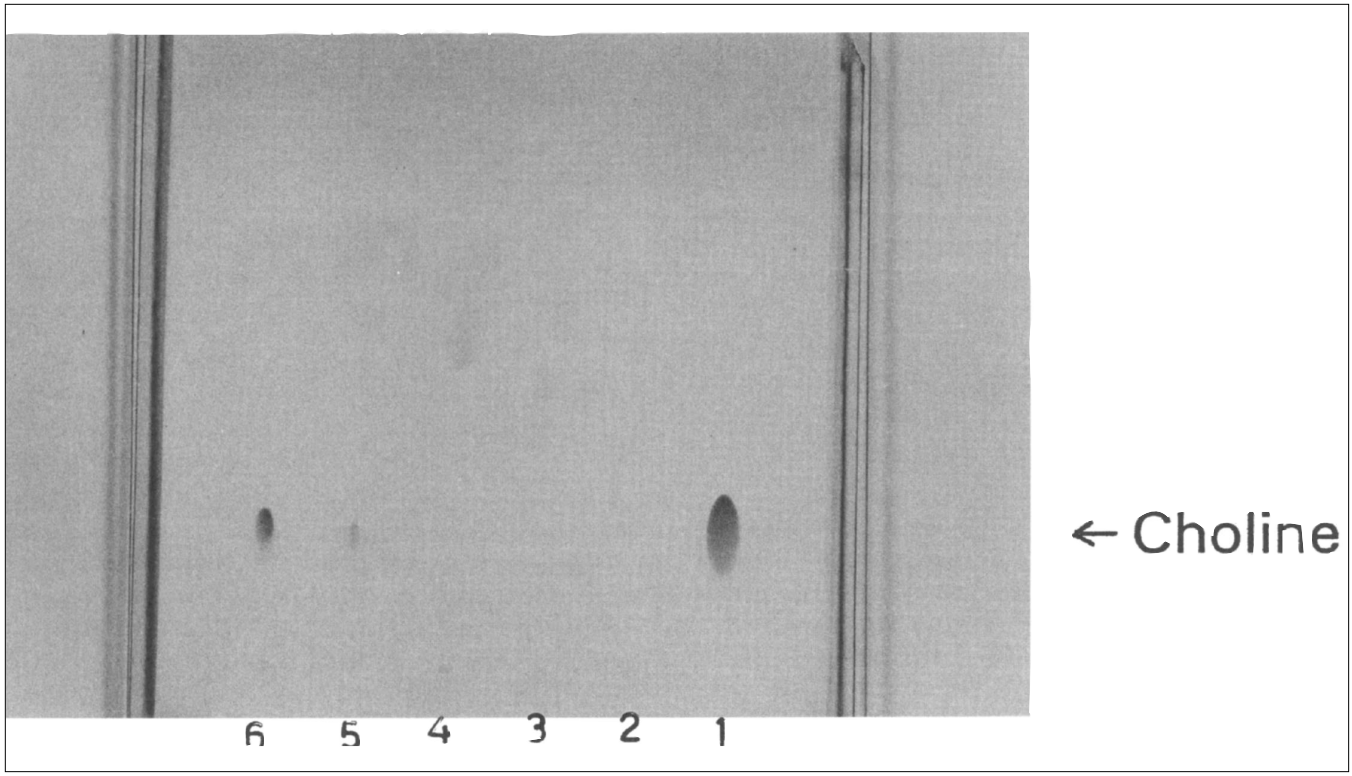


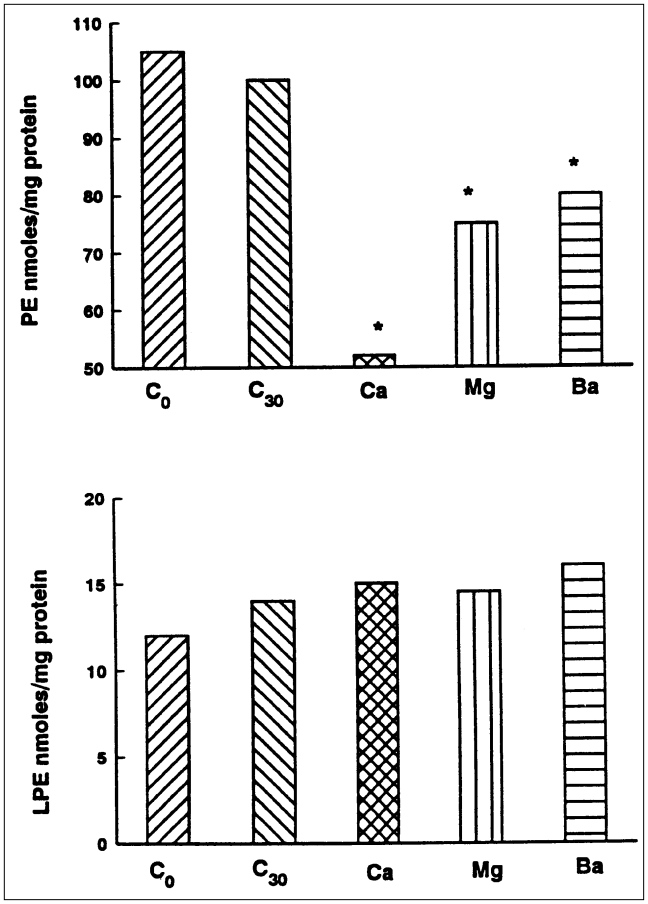
FIG. 6. Choline formation in response to Ca^{2+}, Mg^{2+}, and Ba^{2+} by mitochondrial phospholipase D. The methodology is described in the text. 1. Standard choline; 2. control unincubated mitochondria; 3. control mitochondria incubated for 30 min; 4. incubated with Ca^{2+}; 5. incubated with Mg^{2+}; 6. incubated with Ba^{2+}.

metal ions stimulated the formation, and Ca^{2+} stimulation of the enzyme resulted in the maximum formation of ethanolamine (Fig. 9). PLD-like activity was also checked in presence of phospholipase A_2 inhibitors, namely, CHLP and pBPB. CHLP and pBPB as such stimulated PLD activity and this activity was further enhanced by the presence of Ca^{2+} or Mg^{2+} (data not shown).

DISCUSSION

This study has demonstrated the presence of an active PLD-like activity in the intestinal mitochondria and, using endogenous lipids as substrate, some of the properties of this enzyme were studied. Although PLD activity has been shown in whole cells and subcellular membranes (1,2), very little is known on mitochondrial PLD activity (18,19). PLD activity is usually assayed by PA formation or by measurement of phosphatidylethanol, which is the transphosphatidylation product of PA in the presence of alcohol. In the case of intestinal mitochondrial PLD, no phosphatidylethanol formation could be detected using endogenous substrate, as confirmed by both TLC and HPLC separation. This was further confirmed using mitochondrial enzyme and egg PC as substrate (data not shown). It was found that even in the presence of metal ions, phosphatidylethanol formation could not be detected. Moreover the PA content remained the same whether ethanol was present or not. PA formation by mitochondrial PLD was confirmed by HPLC which gave an identical retention time to that of the authentic standard. Furthermore, analysis of the PA formed showed a phosphate to fatty acid ratio of 1:2 which indicated that PA is the reaction product. That PLD-like enzyme indeed was responsible for PA formation was further confirmed by identifying the other products of reaction, namely, choline and ethanolamine. It may be that either the intestinal mitochondrial PLD-like enzyme does not catalyze transphosphatidylation or this enzyme does not have the transphosphatidylation activity, which was shown to be different from PLD activity in rat brain membranes (34).

Metal ions have been shown to activate or inhibit PLD activity depending on the source of the enzyme (8,35–37). Intestinal mitochondrial PLD-like enzyme was activated by various metal ions, and it was concentration-dependent. Increased PA formation in mitochondria in the presence of metal ions was not due to DAG kinase activity since incubation of mitochondria in presence of DAG and ATP did not increase the PA formation. Moreover, all the DAG added was recovered. Earlier, Rider and Baquet (38) reported that DAG exogenously added to liver microsomes caused 8–10-fold stimulation of inorganic phosphate incorporation in the PA. Kanoh and Akesson (39) also observed a similar stimulation



of the DAG kinase reaction by exogenous DAG. Since mitochondrial PA formation was not influenced by exogenously added DAG, this suggests that PA formation in mitochondria in the presence of metal ions is due to PLD-like activity rather than DAG kinase activity. It has been suggested that PA formed by the action of PLD may be hydrolyzed by the phosphatidate phosphatase to DAG, and this may make the quantitation of the PLD product, PA, difficult. In the case of intestinal mitochondria, it appears that the PA formed is stable, and this may be due to the absence of PA phosphatase activity. This was further supported by our observation that metal ion activation did not increase the level of DAG in mitochondria. Maximal PLD-like activity was obtained at pH 6.5, and activation by metal ions did not alter the pH optimum. This is similar to an earlier report on brain plasma membrane PLD activity (10), but differs from a neutral PLD from rat brain synaptosomal membranes (11). Analysis of mitochondrial lipids suggested that both PC and PE were used as substrate when activated by Mg^{2+} or Ba^{2+}, whereas PE was preferentially used when activated by Ca^{2+}. There are reports on PE preferring PLD activity (1). The basal

FIG. 7. Phosphatidylethanolamine (PE) and lyso PE content of intestinal mitochondria after incubation with Ca^{2+} (1 mM), Mg^{2+} (1 mM), and Ba^{2+} (0.1 mM). PE and lyso PE were derivatized with fluorescamine and separated by thin-layer chromatography and quantitated by spectrofluorimetry as described in the text. Each value represents mean ± SEM of three separate estimations. C_o represents unincubated mitochondria and C_{30} represents mitochondria incubated for 30 min without metal ions. $^*P < 0.05$ for metal ions treated versus control mitochondria (incubated).

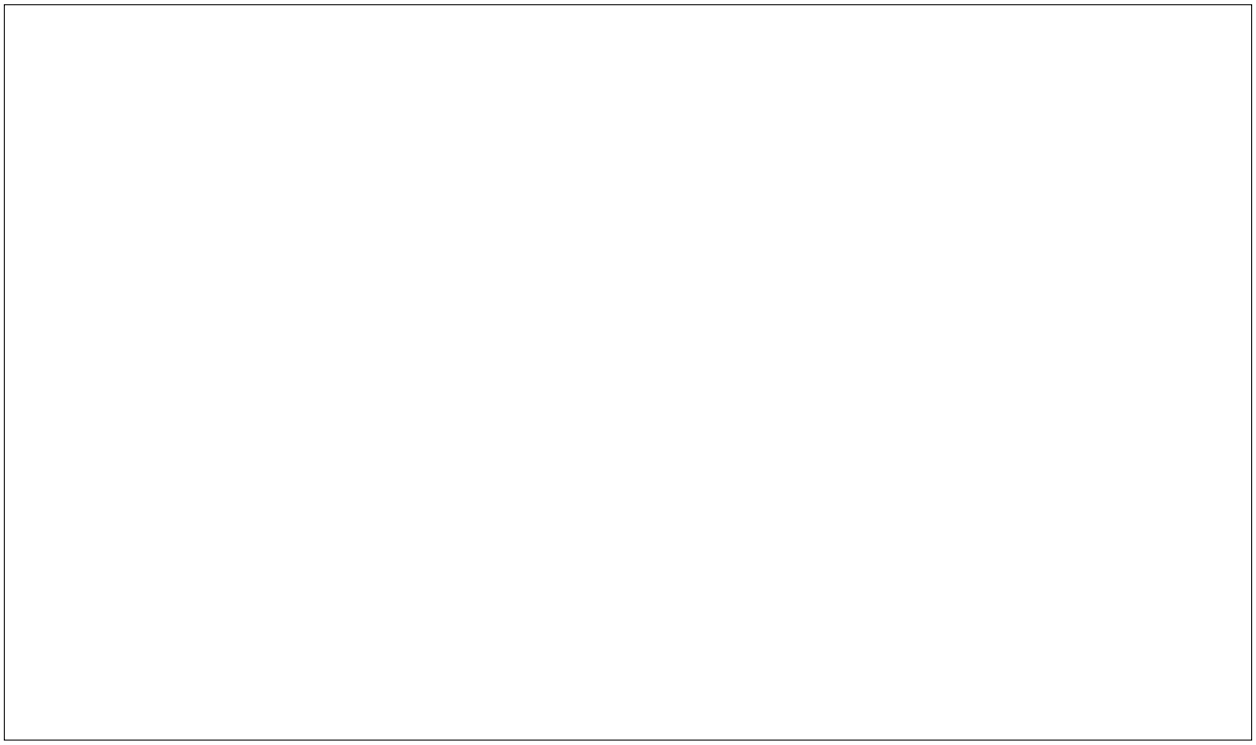


FIG. 8. Ethanolamine formation in response to Ca^{2+}, Mg^{2+}, and Ba^{2+} by mitochondrial phospholipase D. Ethanolamine isolation, fluorescamine derivatization, and separation are described in the text. Lane 1 represents mixture of standard amino acids, glutathione, spermine, and ethanolamine. Lane 2 represents only ethanolamine. Lane 3, unincubated control. Lane 4, control incubated for 30 min. Lane 5, mitochondria incubated with Ca^{2+}. Lane 6, mitochondria incubated with Mg^{2+}. Lane 7, mitochondria incubated with Ba^{2+}.

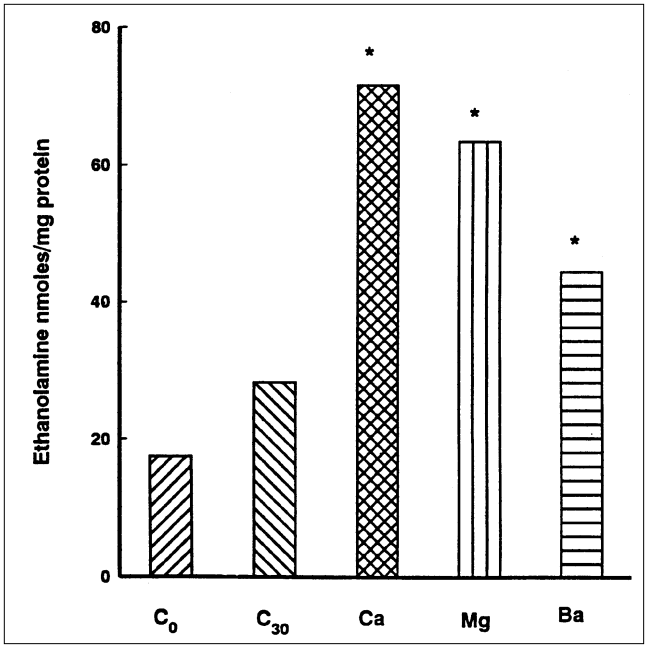


FIG. 9. Effect of metal ions on ethanolamine release from mitochondrial phosphatidylethanolamine. Ethanolamine was separated and quantitated using spectrofluorimetry. Each value represents mean ± SEM of three separate estimations. $^*P < 0.05$.

activity in the mitochondria was not influenced by the presence of EDTA. Our attempts to solubilize the mitochondrial enzyme and partially purify it were not successful since the enzyme was very unstable. Various attempts to stabilize the enzyme was also not successful.

Unlike PLD from other sources, intestinal mitochondrial enzyme does not require sodium oleate for activity. This may be due to large amount of free fatty acids associated with intestinal mucosal membranes (40,41).

It has been suggested that in certain pathological conditions, mitochondria may sequester cytosolic Ca^{2+} in order to prevent cellular damage (12,14). During this process, it may act as a storage organelle for intracellular Ca^{2+}. Mitochondrial membranes are known to have bound Mg^{2+} (42), and the presence of metal ions in close proximity to the mitochondrial PLD might stimulate this enzyme, resulting in alteration of mitochondrial membrane lipids.

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Membrane Lipid Metabolism and Phospholipase Activity in Insect *Spodoptera frugiperda* 9 Ovarian Cells

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ABSTRACT: Although there is increasing use of insect ovarian Sf9 cells for the production of recombinant proteins, namely, via the baculovirus vector expression system, little is known about the lipids in the cell membrane and whether endogenous phospholipases are present for regulation of the cell membrane lipids. In this study, analysis of membrane lipids of Sf9 cells indicated the presence of phosphatidylethanolamine (PE) (diacyl type) and phosphatidylcholine as major phospholipids, followed by phosphatidylserine and phosphatidylinositol (PI), and only trace amounts of ethanolamine plasmalogen. These phospholipids contain high proportions of monoenoic fatty acids, e.g., 16:1 and 18:1, which comprise more than 70% of the total fatty acids although small amounts of polyunsaturated fatty acids such as 18:2 and 20:4 are also present. When Sf9 cells were incubated in a culture medium containing [^{14}C]oleic acid and [^{14}C]arachidonic acid, a large portion of the labels were incorporated into membrane phospholipids. Using [^{14}C]arachidonoyl-phospholipids as substrates for incubation with cell homogenate and subcellular fractions, results indicate the presence of a $Ca2^{+}$-independent phospholipase A (PLA_2) in the Sf9 cell cytosol fraction. This PLA_2 shows a high preference for hydrolysis of PE and is active at a pH range of 7–9. Unlike the brain cells which contain active phospholipase C (PLC) specific for phosphatidylinositol, only limited amount of diacylglycerol (DAG) was released from [^{14}C]arachidonoyl-PE in the Sf9 cells. Taken together, this study demonstrates active metabolism of membrane phospholipids in Sf9 cells, most likely mediated by acyltransferases and PLA_2. Furthermore, despite the absence of PLC for PI, limited amount of DAG could be generated through hydrolysis of PE.

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Recent advances in molecular biological techniques have focused on the use of insect ovarian *Spodoptera frugiperda* (Sf9) cells for the production of recombinant proteins, mainly *via* the baculovirus vector expression system (1). There are several advantages in the use of these cells in biotechnology, e.g., their ability to grow in a chemically defined medium at ambient temperature, their rapid growth rate, and possibility of large-scale production of cells grown in suspension. In the biotechnology of mass protein production, it is important to consider the ability of the host cells to carry out post-translational modifications and processing, e.g., phosphorylation, acylation, glycosylation and in the case of membrane proteins, translocation and incorporation into the membrane. In the study by De Carvalho *et al.* (2), there is evidence that expression of the 84 kDa cytosolic phospholipase A_2 (PLA_2) in Sf9 cells resulted in a phosphorylated protein which is actually the active form of the enzyme (3). The study of Leimer *et al.* (4) demonstrated that overexpression of rat synaptophysin in Sf9 cells resulted not only in the synthesis of the complete polypeptide which is a *N*-glycosylated integral membrane protein, but also incorporation of the protein into membrane vesicles. Other studies also show that Sf9 cells are capable of synthesizing functionally active proteins which are subsequently incorporated into the plasma membranes as channels (5) or receptors (6,7). A recent review on the G-protein-coupled receptors in insect cells gave evidence suggesting that these cells are suitable for large-scale expression and characterization of cloned receptor genes (8)

In order to better understand the Sf9 cells expression system, it is important to know the characteristic metabolic activities in these cells. For example, it would be of interest to obtain information on the membrane lipid composition and the enzymes metabolizing the lipids in these cells. It would be of interest to find out whether different phospholipases are present in these cells and whether second messengers, e.g., inositol (1,4,5) trisphosphate and diacylglycerol (DAG), are released upon stimulation of the cell surface receptors (9). These second messengers are important in mediating intracellular calcium homeostasis and in turn, responsible for regulation of a number of cellular metabolic pathways (10).

In this study, the lipids present in Sf9 cells were character-

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Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; DAG, 1,2-diacylglycerol; DITC, homogenate of immortalized astrocytes; FAME, fatty acid methyl ester; FBS, fetal bovine serum; FFA, free fatty acid; HPTLC, high-performance thin-layer chromatography; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PE_{p1}, ethanolamine plasmalogen; PI, phosphatidylinositol; PS, phosphatidylserine; PLA_2, phospholipase A_2; PLC, phospholipase C; STE, sucrose-Tris-EDTA buffer; TAG, triacylglycerol; UV, ultraviolet.

ized, and metabolism of labeled fatty acids and labeled phospholipids was examined. We obtained evidence that the insect Sf9 cells can actively take up fatty acids from the culture medium and that the cell cytosol contains an active calcium-independent PLA_2.

MATERIALS AND METHODS

Cell culture and subcellular fractionation. Sf9 cells were grown in stationary T175 culture flasks at 28°C in TNM-FH medium supplemented with 10% fetal bovine serum (FBS) (1). The cells were subcultured twice a week, and experiments were carried out shortly after cells reached confluency. In some experiments, cells were harvested and subjected to a subcellular fractionation protocol for isolation of crude mitochondrial and microsomal fractions. Briefly, cells were homogenized in an STE buffer containing 0.32 M sucrose, 50 mM Tris, and 1 mM acid free EDTA (pH 7.4) and centrifuged at $1,000 \times g$ to remove unbroken cells and nuclei. The suspension was then centrifuged at $20,000 \times g$ for 30 min at 4°C. The pellet containing the crude mitochondrial membrane fraction was resuspended in sucrose-Tris-EDTA (STE) buffer and stored at −20°C until further use. The supernatant was centrifuged at $100,000 \times g$ for 60 min to yield a microsomal pellet which was suspended in STE buffer and stored at −20°C. The remaining supernatant was the cytosol fraction.

Analysis of cell membrane lipids and their acyl group composition. Cell samples containing 1×10^7 cells were collected, centrifuged at 800 rpm for 5 min, and resuspended in 2 mL Hank's balanced salt solution. Cells were disrupted using a glass-homogenizer, and protein content in the cell homogenates was determined using the Bio-Rad procedure (11) with bovine serum albumin (BSA) as a protein standard. Protocols for lipid extraction and analysis were essentially those described previously by Sun (12). Lipids from cell homogenate were extracted by adding 4 mL of chloroform/methanol (2:1, vol/vol) to a 1 mL aliquot of cell homogenate followed by vortexing for 30 s. After a low-speed centrifugation ($800 \times g$), the lower organic phase was filtered through an anhydrous Na_2SO_4 column, dried under N_2, and reconstituted in 1 mL of chloroform/methanol (2:1, vol/vol).

For separation of the neutral lipids, a 0.5 mL aliquot of the reconstituted organic phase was applied to high-performance thin-layer chromatography (HPTLC) plate (silica gel HP-K, 10×10 cm, Whatman, Hillsboro, OR) and developed twice in a solvent system containing hexane/diethyl ether/acetic acid (85:15:2, by vol). Using this solvent system, neutral lipids, including triacylglycerol (TAG), free fatty acid (FFA), and diacylglycerol (DAG)/cholesterol were separated from phospholipids which remained at the origin. For visualization of lipid bands, the HPTLC plate was either exposed to iodine vapors (for counting of radioactivity) or sprayed with 1% 2′,7′-dichlorofluorescein in methanol and viewed under an ultraviolet (UV) lamp (for analysis of fatty acids).

The phospholipids that remained at origin of the HPTLC plate were recovered by suspending the mixture in a two-phase system containing chloroform/methanol/1% acetic acid (8:4:3, by vol). The phospholipids in the organic phase were recovered and separated by a two-dimensional HPTLC system (12). The first solvent system contained chloroform/methanol/acetone/16 N NH_4OH (70:40:10:10, by vol). After solvent development, the plates were thoroughly dried by blowing with an air-gun and were exposed to HCl fumes for 3 min. This procedure separates the plasmalogens from the diacyl-phospholipids. After removing the residual HCl by blowing, the HPTLC plates were turned 90° and developed in the second solvent system containing chloroform/methanol/acetone/acetic acid/0.1 M ammonium acetate (70:30:27.5:2.3:5, by vol). After solvent development, plates were either exposed to iodine vapors or sprayed with 2′,7′-dichlorofluorescein, and lipid spots corresponding to phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC), ethanolamine plasmalogen (PE_{pl}) and phosphatidylethanolamine (PE) were removed from the plate by scraping. Samples were either used for measurement of radioactivity (iodine) or further processed for fatty acid analysis (2′,7′-dichlorofluorescein).

For analysis of the acyl group composition of phospholipids, samples were subjected to base methanolysis using 1 mL of 0.5 N NaOH in methanol together with heptadecanoic acid methyl ester (17:0 FAME) as internal standard (12). The fatty acid methyl esters (FAME) were recovered by partitioning the reaction mixture into the chloroform/methanol/H_2O system. The organic solvent containing the FAME was removed and solvent evaporated under N_2. In some instances, FAME were further purified by HPTLC using the hexane/ether/acetic acid system. FAME were analyzed by gas–liquid chromatography using a Hewlett-Packard Gas Chromatograph (model 5890A; Wilmington, DE) equipped with a flame-ionization detector and a plotter-integrator unit and a fused-silica capillary column (0.32 mm i.d. × 30 m length; Supelco Co., Bellefonte, PA).

Uptake of labeled oleic acid into cell membrane lipids. In order to determine whether Sf9 cells can take up labeled fatty acids from the culture medium, the solvent used for dissolving [1-^{14}C]oleic acid (1 mCi/mL, specific activity 3000 Ci/mmol; New England Nuclear, Boston, MA) was evaporated under N_2, and the fatty acid was suspended in TNM-FH medium containing 2% FBS and vortexed vigorously. Sf9 cells were harvested and were added to the medium containing the label (0.1 μCi/mL) and incubated for 30 min. After incubation, the reaction was terminated by adding 4 mL of chloroform/methanol (2:1, vol/vol) to 1 mL of the incubation mixture. Lipids were extracted and analyzed as described above. Lipid spots were removed from HPTLC plates to scintillation vials for measurement of radioactivity by a Beckman LS 5800 scintillation spectrometer (Fullerton, CA).

Preparation of labeled substrates. PC, PS, PE, and PI labeled with [^{14}C]arachidonic acid (AA) (20:4) were synthesized in our laboratory by incubating the rat pheochromocytoma cells (PC-12) with [1-^{14}C]AA (2 μCi/60 mm dish) for 6 h as described elsewhere (13). [1-^{14}C]Oleate-labeled PE and PC substrates were prepared by incubating Sf9 cells with

[1-^{14}C]oleic acid for 6 h. [1-^{14}C]Palmitate-PC was prepared by incubating PC-12 cells with [1-^{14}C]palmitic acid for 6 h. Briefly, after incubation with labeled fatty acids, cells were harvested and lipids extracted and separated by two-dimensional HPTLC system as described above (12). After separation, the HPTLC plates were sprayed with 2′,7′-dichlorofluorescein and visualized under a UV lamp. Individual lipid spots were transferred to test tubes, and the labeled phospholipids were recovered in the lower organic phase after partitioning the sample into a two-phase system by addition of 4 mL chloroform/methanol (2:1, vol/vol) and 1 mL of 0.1 N NaOH. For estimation of the specific radioactivity of the lipid substrates, a separate dish of cultured cells was used and lipids were separated by the same two-dimensional HPTLC protocol. The amount of fatty acids from individual phospholipids was determined together with 17:0 methyl ester as an internal standard. From this assay, it is possible to determine specific radioactivity of the fatty acids in each phospholipid.

Assay of phospholipase A_2 (PLA_2) and phospholipase C (PLC) activity. For assay of PLA_2 and PLC activities in Sf9 cell homogenates and subcellular fractions, solvent for dissolving the labeled phospholipids (5000 to 10,000 dpm) was evaporated under N_2 and mixed with 100 µL of the incubation buffer containing 1 mM EGTA, 1 mM EDTA, 6 mM $CaCl_2$, 1 mg/mL BSA, and 50 mM HEPES (pH 7.4). Enzyme reaction was initiated by adding 100 µL of the cell homogenate or subcellular fractions, and incubation was carried out at 37°C for 30 min or time indicated. Reaction was terminated by adding 4 mL of chloroform/methanol (2:1, vol/vol) followed by 0.8 mL of 0.1 N HCl. Lipids in the lower organic phase together with DAG and FFA standards were applied to HPTLC plates and separated using a solvent system containing hexane/ethyl ether/acetic acid (85:15:2, by vol). After solvent development, the HPTLC plates were exposed to iodine vapors for visualization. Lipid bands corresponding to phospholipids (origin), DAG, and FFA were identified and transferred to scintillation vials for measurement of radioactivity. In each experiment, a negative control ($t = 0$) was carried out by mixing homogenate with incubation buffer and label and immediately stopping the reaction with 4 mL of chloroform/methanol (2:1, vol/vol). PLA_2 activity was expressed as nmol of FFA released per 100 µg protein per unit time. Similarly, PLC activity was expressed as nmol of DAG released per 100 µg protein per unit time.

RESULTS

Membrane lipids and acyl group composition in Sf9 cells. Analysis of the lipids in Sf9 cells indicated a phospholipid profile similar to those present in mammalian cells (Fig. 1). The major phospholipid is the diacyl form of PE (41.8%) followed by PC (36.4%), PI (14.5%), and PS (7.3%). Despite the fact that PE is the major phospholipid, only trace amounts of PE_{pl} are present. Data in Figure 1 further show that noticeable amounts of sphingomyelin and cardiolipin are present in these cells.

An example of the elution profile of FAME derived from total phospholipid extract of Sf9 cells is shown in Figure 2.

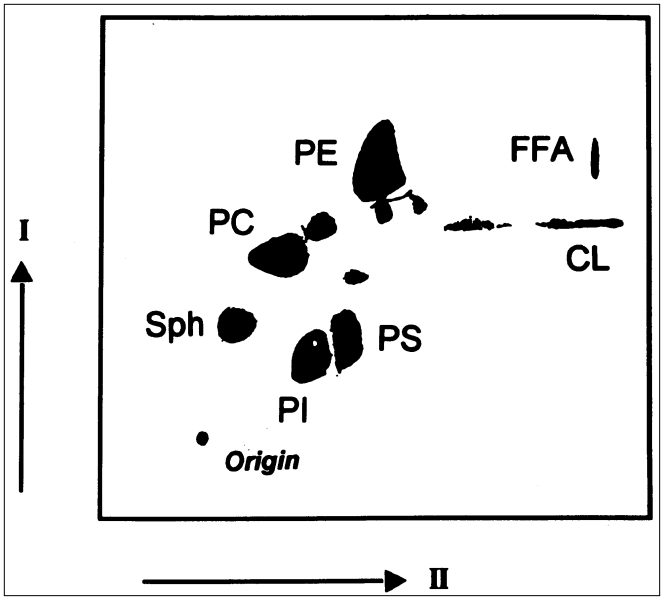

FIG. 1. A typical picture depicting the phospholipids of *Spodoptera frugiperda* cells. *Spodoptera frugiperda* cells (1×10^7 cells) were harvested, washed, and lipids extracted by chloroform/methanol (2:1, vol/vol). The lipid extract was separated by two-dimensional high-performance thin-layer chromatography according to a procedure described by Sun (12); PS, phosphatidylserine; PI, phosphatidylinositol; Sph, sphingomyelin; CL, cardiolipin; PC, phosphatidylcholine (split into two spots); PE, phosphatidylethanolamine; FFA, free fatty acid. Note: Minor spots in the chromatogram were not identified.

These cells exhibit a relatively simple fatty acid profile with monounsaturated fatty acids, e.g. 16:1, 18:1, constituting more than 70% of the total fatty acids. Saturated fatty acids include 16:0, 18:0, and 20:0. (The identity of 20:0 was confirmed by co-chromatography with a known standard.) Polyunsaturated fatty acids such as 18:2 and 20:4 are present at low levels. When the acyl group composition of individual phospholipids was analyzed, both PE and PC constitute high proportions of 16:1 and 18:1 whereas PS/PI show high proportions of 18:0 and 18:1 (Table 1). Interestingly, 20:0, a unique fatty acid of Sf9 cells, is concentrated in the PS/PI fraction (10.3% as compared to 3.1% in the total homogenate).

Uptake and incorporation of labeled oleic acid by Sf9 cells. The ability of Sf9 cells to take up and incorporate [1-^{14}C]oleic acid into membrane phospholipids and other glycerolipids was examined. Results show that labeled oleic acid was taken up from the culture medium and incorporated into phospholipids as well as TAG. The incorporation of labeled oleic acid into TAG reached a plateau by 2 h, whereas the incorporation into phospholipids did not attain a plateau until after 4 h (Fig. 3). When the lipids were separated by two-dimensional HPTLC, incorporation of labeled oleate was highest in PE, followed by PC and very low in PI and PS (Fig. 4).

We further examined the uptake profile of [^{14}C]oleic acid among the glycerolipids in two subcellular membrane fractions of Sf9 cells. In this experiment, cells were incubated with [^{14}C]oleic acid (0.1 µCi/mL) for 6 h. After harvesting the cells,

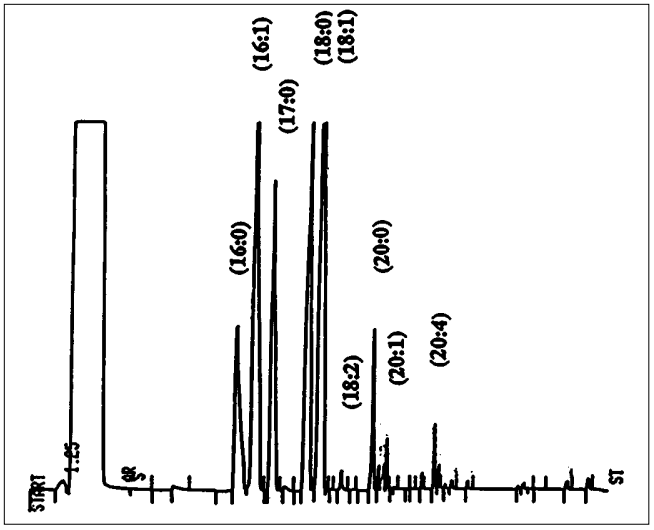

FIG. 2. The fatty acid profile of total phospholipids from *Spodoptera frugiperda* cells. Phospholipids were separated from other lipids by one-dimensional high-performance thin-layer chromatography. Fatty acids from the phospholipids (together with a known amount of 17:0 methyl ester) were converted to fatty acid methyl esters by basic base-catalyzed methanolysis and analyzed by gas–liquid chromatography.

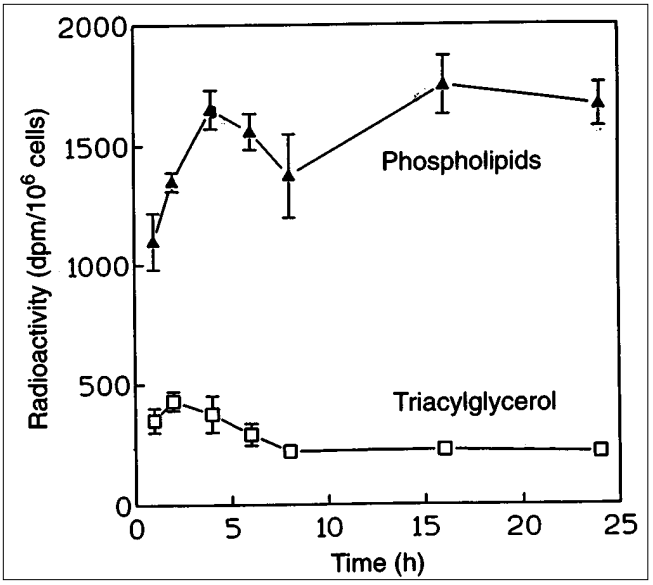

FIG. 3. Time course of [^{14}C]oleic acid uptake into phospholipids and triacylglycerol of *Spodoptera frugiperda* cells. *Spodoptera frugiperda* cells (1×10^6 cells per 35-mm dish) were incubated in 1 mL of TNM-FH medium containing [1-^{14}C]oleic acid (0.1 μCi/dish). At specified time, cells were washed twice, each with 1 mL of Hank's balanced salt solution, and the lipids were extracted with chloroform/methanol (2:1, vol/vol). Lipids were separated by the one-dimensional high-performance thin-layer chromatography to give the triacylglycerol band and phospholipids (at origin). Results are mean ± SD from three samples for each time point. Two other experiments showed similar results.

they were homogenized in a sucrose-Tris buffer. Cell homogenate was subjected to differential centrifugation to yield a crude mitochondria and a microsomal fraction. The membrane lipids were extracted and were separated into neutral lipids and phospholipids using the HPTLC protocols as previously described. In the crude mitochondria fraction, 15% of the label was in neutral lipids and 85% in the phospholipids. Analysis of the phospholipids indicated a labeling order of PE > PC > PI = PS = PA (Fig. 5). When the labeling pattern of glycerolipids in microsomes was compared to the mitochondrial fraction, glycerolipids in the microsomes indicated a higher proportion of radioactivity in TAG and polyphosphoinositide and a lower proportion in PE (Fig. 5).

TABLE 1
Fatty Acid Composition (wt%) of Total and Individual PL in *Spodoptera frugiperda* Cells[a]

Fatty acids	PL	PE	PC	PS + PI
16:0	7.6	3.3	5.4	4.6
16:1	31.3	23.4	26.4	18.0
18:0	14.6	9.7	11.1	35.1
18:1	40.3	55.5	52.4	26.8
20:0	3.1	4.6	2.7	10.3
20:1	0.9	3.0	1.7	2.5
20:4	1.2	0.6	0.3	2.7

[a]Lipid extract from *Spodoptera frugiperda* cells was separated by high-performance thin-layer chromatography first by the one-dimensional system to obtain total phospholipids (PL) and followed by a two-dimensional system for individual PL. PL were subjected to base-catalyzed methanolysis, and the fatty acid methyl esters were subsequently analyzed by gas–liquid chromatography. Data are expressed as weight percentage of total fatty acids (average of two samples from a typical experiment); PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol.

Using Sf9 cell homogenate, we compared the labeling profile due to incorporation of [^{14}C]AA and [^{14}C]oleic acid into the glycerolipids. Data in Figure 6 show that labeled AA was

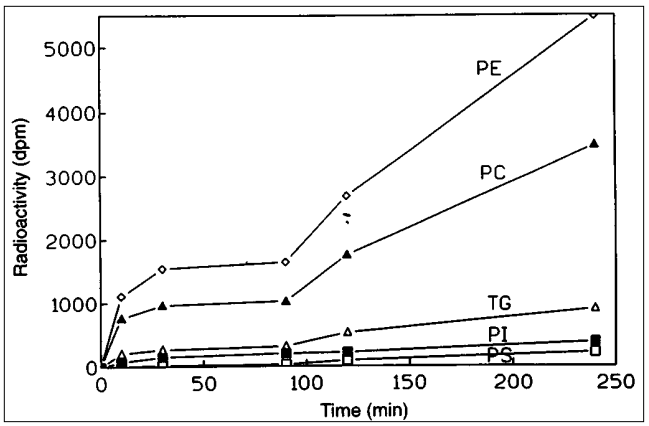

FIG. 4. Time course of [^{14}C]oleic acid incorporation into individual phospholipid species and triacylglycerol of *Spodoptera frugiperda* cells. Incubation conditions were the same as in Figure 3 except that lipids were separated by one-dimensional high-performance thin-layer chromatography (for separation of triacylglycerol and phospholipids) followed by recovering the phospholipids for two-dimensional high-performance thin-layer chromatography as described in the text. Results are depicted as radioactivity of lipids (average of two samples) recovered from 1×10^6 cells.

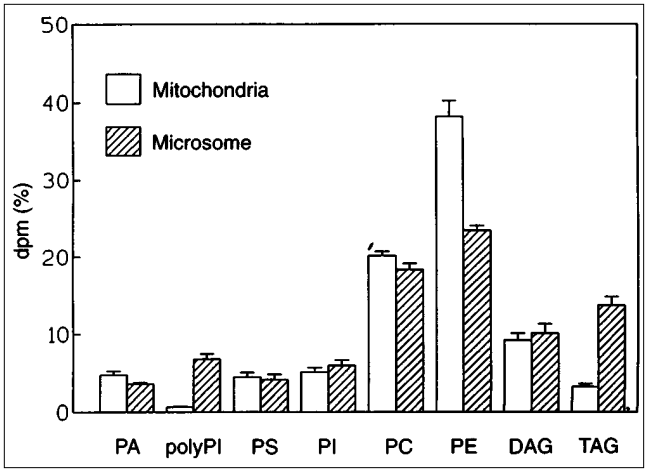

FIG. 5. Percentage distribution of [^{14}C]oleic acid among glycerolipids in mitochondrial and microsomal fractions of *Spodoptera frugiperda* cells. *Spodoptera frugiperda* cells (10×10^8) were incubated with [^{4}C]oleic acid for 6 h. After labeling, cells were washed and subjected to subcellular fractionation to yield the mitochondrial and microsomal fractions. Analysis of lipids from the subcellular fractions was similar to that described in Figure 4. Data represent percentage distribution of radioactivity among glycerolipids and are mean ± SD from three samples; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol. See Figure 1 for other abbreviations.

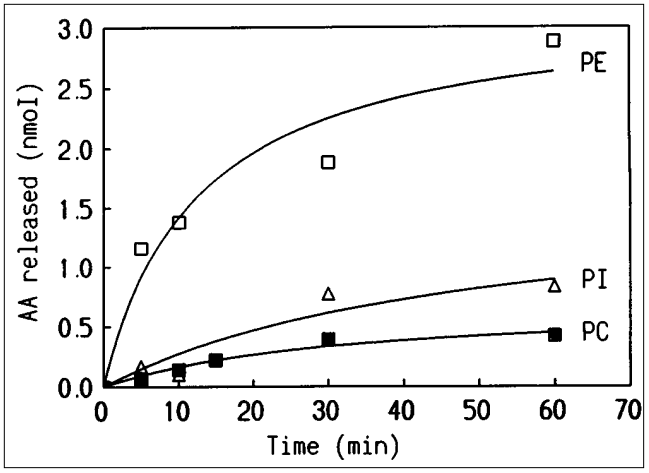

FIG. 7. Time course of arachidonic acid (AA) release from *Spodoptera frugiperda* cell homogenate after incubation with different [^{14}C]20:4-phospholipids. The incubation mixture contained cell homogenates (100 µg protein) and labeled phospholipids in a buffer made of bovine serum albumin (1 mg/mL), $CaCl_2$ (6 mM), EGTA (1 mM), EDTA (1 mM) and HEPES (pH 7.4). Incubation was carried out at 37°C for different time periods. After termination of reaction, labeled AA were separated by one-dimensional high-performance thin-layer chromatography. Phospholipase A_2 activity is represented by the release of labeled AA (nmol/100 µg protein) during the specified times. Results are average values of two samples from a typical experiment. See Figure 1 for other abbreviations.

more actively incorporated into PI and PC and less into PE and TAG as compared to the pattern due to labeled oleic acid.

Test for PLA$_2$ and PLC activity in Sf9 cells. In order to test for the presence of PLA$_2$ and PLC in Sf9 cells, cell homogenates were incubated with [^{14}C]20:4-PC, PE, PI, and PS at 37°C for 30 min. These lipid substrates were prepared by incubating PC-12 cells with [^{14}C]AA as described in the Materials and Methods section. Data in Figure 7 show the time-dependent release of labeled FFA upon incubation of Sf9 cell homogenate with labeled PE, PC, and PI. Results show that the release of labeled AA from PE was several-fold higher than that of labeled PI and PC (Fig. 7).

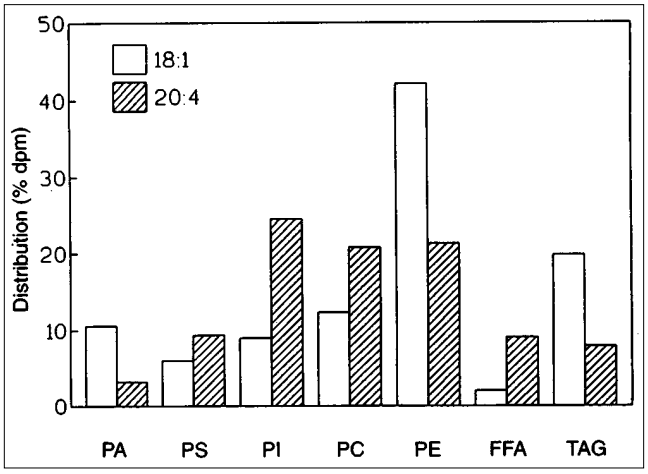

FIG. 6. Percentage distribution of [^{14}C]oleic acid and [^{14}C]arachidonic acid in glycerolipids of *Spodoptera frugiperda* cells. *Spodoptera frugiperda* cells (1×10^6 cells per 35-mm dish) were incubated in 1 mL of TNM-FH medium containing the respective labeled fatty acids. Analysis of lipids was similar to the described in Figure 4. Data are percent distribution of radioactivity among glycerolipids and are average from two samples. Note: Radioactivity in the free fatty acid (FFA) fraction depicts the amount of radioactivity of free fatty acids retained within the cells. See Figures 1 and 5 for other abbreviations.

In a separate experiment, the ability of Sf9 cell homogenate to hydrolyze different [^{14}C]20:4-phospholipids was compared with the homogenate of immortalized astrocytes (DITNC) derived from rat brain (14). Data in Table 2 show that Sf9 cell homogenates preferentially hydrolyzed [^{14}C]20:4-PE over other labeled phospholipids, whereas DITNC cells show a clear order of PE > PC > PI > PS. Besides the release of FFA, incubation of DITNC cells with labeled PI resulted in a large release of labeled DAG. In fact, there was more labeled DAG released than

TABLE 2
Incubation of [14C]20:4-PL with *Spodoptera frugiperda* Cell Homogenate[a]

	Sf9		DITNC	
	FFA	DAG	FFA	DAG
[^{14}C]20:4-PE	13.9	0.19	0.48	0.077
[^{14}C]20:4-PI	0.17	n.d.	0.13	0.56
[^{14}C]20:4-PI	0.17	n.d.	0.016	0.005
[^{14}C]20:4-PC	0.071	0.026	0.26	0.037

[a]*Spodoptera frugiperda* cell homogenate (100 µg protein) was incubated with individual labeled PL at 37°C for 30 min as described in text. Values are nmol of substrates free fatty acid (FFA) and diacylglycerol (DAG) released per 100 µg protein per 30 min and are average of duplicate incubations from a typical experiment; n.d., not detected; DITNC, homogenate of immortalized astrocytes. See Table 1 for other abbreviations.

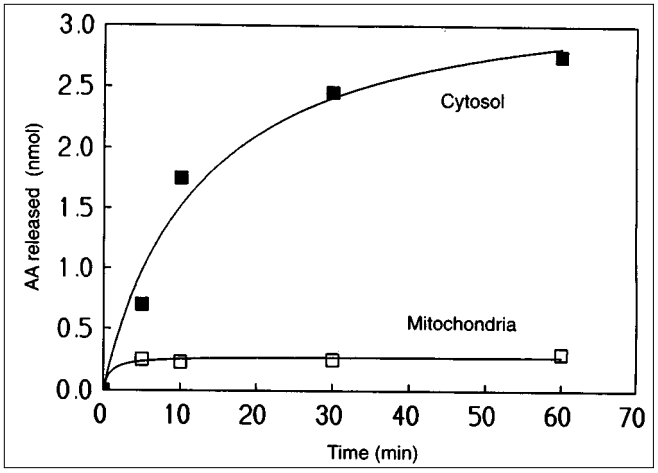

FIG. 8. Measurement of phospholipase A_2 activity in mitochondrial and cytosol fractions of *Spodoptera frugiperda* cells. Subcellular fractions were prepared as described in the text. Samples were incubated with [^{14}C]20:4-phosphatidylethanolamine as described in Figure 7. Data are expressed as nmol of fatty acids released per 100 µg of protein and are averages from two samples.

FFA. On the other hand, the only labeled phospholipid substrate which can give an observable labeled DAG in Sf9 cells is [^{14}C]20:4-PE (Table 2).

Subcellular localization of phospholipases. In this experiment, Sf9 cells (3×10^7 cells) were washed, homogenized, and subjected to differential centrifugation to yield the mitochondrial and cytosolic fractions. Aliquots of these fractions were used in a time-course study with [^{14}C]20:4-PE. Surprisingly, there was a 10-fold higher release of labeled 20:4 from PE by the cytosol fraction compared to the mitochondrial fraction (Fig. 8). When the cytosol fraction was incubated with different labeled phospholipids, a similar substrate profile with preference on [^{14}C]arachidonoyl-PE was observed (data not shown). We also tested the ability of cell cytosol to hydrolyze [^{14}C]oleoyl-PE and PC as well as [^{14}C]palmitoyl-PE. However, none of these substrates showed activity comparable to the [^{14}C]arachidonoyl-PE (data not shown).

Calcium and pH on cytosolic PLA_2 in Sf9 cells. In order to test for Ca^{2+} dependency, cell cytosol was incubated with [^{14}C]20:4-PE in a buffer containing either EGTA or EGTA with Ca^{2+} giving final [Ca^{2+}] from 0 to 2.7 mM, respectively. To our surprise, the release of FFA from [^{14}C]20:4-PE was highest in the samples with EGTA alone. Addition of Ca^{2+} to the incubation system did not cause an obvious inhibition until concentrations well over 2 mM (data not shown). When aliquots of cytosol (100 µg protein) were incubated with [^{14}C]20:4-PE in a Ca^{2+}-free buffer ranging from pH 5 to 9, PLA_2 activity was found at pH 7 and over (data not shown).

DISCUSSION

In this study, we present novel information on the phospholipids and their fatty acid composition in insect ovarian Sf9 cells. Unlike mammalian cells which show a high proportion of PC as the predominant phospholipid and where the PE was comprised of both the diacyl and alkenylacyl form, insect Sf9 cells contain a high proportion of PE which is present mainly in the diacyl form. Analysis of the fatty acids of phospholipids in Sf9 cells revealed that these cells have a fatty acid pattern comprising high proportions of 16:1 and 18:1 with only low proportions of polyunsaturated fatty acids.

When labeled fatty acids were added to the culture medium, it is clear that Sf9 cells can take up and utilize exogenous fatty acids for their cell membrane lipids. Nevertheless, it is not possible to predict from results of this study whether the incorporation is through the lysophospholipid/acyl–CoA acyltransferase or the *de novo* biosynthesis pathway.

Incubation of Sf9 cell homogenates with [^{14}C]20:4-phospholipids resulted in the release of labeled fatty acid, suggesting the presence of PLA_2 in these cells. Under these *in vitro* assay conditions, this PLA_2 shows a high preference for PE. Incubation of labeled PE also resulted in the release of a small amount of DAG suggesting the presence of a PE-specific PLC in Sf9 cells. In the brain cells, incubation of labeled PI resulted in the release of labeled FFA and DAG, indicating the presence of both PLA_2 and PLC. The lack of a PLC toward labeled PI in Sf9 cells is a strong indication that these cells lack the polyphosphoinositide signaling cascade. So far, the only agonist reported for these cells is octopamine, a biogenic amine known to couple to the adenylate cyclase system (15,16). Despite the absence of PI–PLC in Sf9 cells, results show that some DAG may be derived from hydrolysis of PE instead. However, more studies are needed to test whether the DAG pool generated from PE is responsible for regulation of the protein kinase C in these cells.

Besides showing a preference for the hydrolysis of [^{14}C]20:4-PE, the PLA_2 in Sf9 cells present in the cytosolic fraction is Ca^{2+}-independent and exhibits activity at the alkaline pH range. A Ca^{2+}-independent PLA_2 is found in the fat body of the tobacco hornworm *Manduca sexta* (17). Although little is known about PLA_2 in insect cells, Ca^{2+}-independent PLA_2 is widespread in the cytosol of many mammalian systems (18–20). Because Sf9 cells grow rapidly, it is possible that active PLA_2 is required for regulating membrane phospholipid turnover in these cells. Obviously, more studies are needed to further elucidate the mechanism for activation of this cytosolic PLA_2 in the insect Sf9 cells.

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Biosynthesis of Medium-Chain Triacylglycerols and Phospholipids by HepG-2 Cells

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ABSTRACT: In an attempt to understand the metabolism by the liver of fatty acids (FA) of different chain length, we have studied the incorporation of [1-^{14}C]-labeled C_2, C_8, C_{10}, C_{12}, and C_{16} into cellular lipids by HepG-2 cells. Over 90% of the radiolabeled FA were detected in phospholipids (PL) and triacylglycerols (TAG). The incorporation of C_{12} and C_{16} was three to four times higher than that of C_8 and C_{10} (and reached 35 nmoles per mg protein after 1.5 h). The radioactivity of C_2, C_8, and C_{10} was recovered mainly in PL. C_{12} and C_{16} were incorporated at approximately equal amounts into PL and TAG. The radioactivity of both C_2 and C_8 was recovered exclusively in long-chain FA, suggesting oxidation of C_8 into C_2 units prior to FA synthesis. C_{10} likewise yielded mainly long-chain FA. However 10% of unchanged C_{10} was found in PL and up to 30% in TAG. ^{14}C-C_{12} was largely incorporated unchanged. Under these conditions, the presence of C_{10} and C_{12} in PL and TAG was shown also by gas–liquid chromatography. In the presence of either C_2, C_8, or C_{10}, up to 30% of ^{14}C-monounsaturated FA were detected in PL and TAG. With C_{12} and C_{16}, the fraction of ^{14}C-monounsaturated FA was much smaller suggesting that extensive desaturation occurred during *de novo* synthesis.
Lipids 32, 489–495 (1997).

The influences of both enterally and parenterally administered triacylglycerols (TAG) composed of fatty acids (FA) of different chain lengths on serum lipids and lipoproteins and on biliary lipids have been the subject of many investigations in recent years (1–8). In these dietary studies, the TAG were composed of long-chain fatty acids (LCFA), i.e., FA with 14–18 carbons (1–9). Recently, the effects of medium-chain TAG (MCT) were studied (10–14). These studies demonstrated that varying the FA composition of dietary TAG affected plasma lipid composition very markedly. Specifically, feeding healthy volunteers with MCT resulted in a three-fold increase of plasma TAG level and a significant alteration of the FA composition of TAG in comparison to volunteers fed with long-chain TAG (LCT) (13,14).

In our recent clinical studies, we have observed marked differences in the lipid composition of blood and bile following short-term infusion (24 h) of TAG of different chain lengths (7) while a mixture of MCT and LCT (50%:50%, w/w) given to patients prior to cholecystectomy caused an increase in the concentrations of biliary phospholipids (PL) and cholesterol; less pronounced effects were observed with LCT. The FA composition of biliary PL was similar in patients treated with the different lipid emulsions. In an attempt to understand the results of the latter studies, we have carried out a systematic *in vitro* study presented in this paper on the metabolism of FA of different chain length by liver cells. Since lipid emulsions are rapidly hydrolyzed in the circulation (2), a large fraction of FA uptake by the liver involves unesterified, albumin-bound LCFA and free FA of short- and medium-chain (SCFA and MCFA) length (15). SCFA and MCFA may also reach the liver *via* the portal circulation when TAG containing these FA are fed.

HepG-2 cells were chosen since these cells were found to express a wide variety of liver-specific functions (16). The cells were incubated with different radiolabeled (^{14}C) FA and their incorporation into cellular lipids was followed. C_{12} and C_{16} were incorporated unchanged into cellular TAG and PL. By contrast, octanoic acid (C_8) was apparently utilized only after complete degradation into C_2 units, which were then used for *de novo* synthesis of LCFA. Decanoic acid (C_{10}) was metabolized by both these pathways: it was detected as unchanged ^{14}C-C_{10} in cellular lipids, mainly TAG, or as ^{14}C-LCFA.

MATERIALS AND METHODS

Materials. Minimal essential Eagle's medium (MEM), fetal calf serum (FCS) and penicillin–streptomycin solution were obtained from Biological Industries (Beth Haemek, Israel).

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Abbreviations: BSA, bovine serum albumin; C_2, acetic acid; C_8, octanoic acid; C_{10}, decanoic acid; C_{12}, dodecanoic acid (lauric acid); C_{16}, hexadecanoic acid (palmitic acid); FA, fatty acids; FCS, fetal calf serum; GLC, gas–liquid chromatography; LCFA, long-chain fatty acids; LCT, long-chain triacylglycerols; MCFA, medium-chain fatty acids; MCT, medium-chain triacylglycerols; MEM, minimal essential Eagle's medium; PBS, phosphate-buffered saline; PL, phospholipids; SCFA, short-chain fatty acids; TAG, triacylglycerols; TLC, thin-layer chromatography; UV, ultraviolet.

[1-^{14}C] Sodium acetate (52.1 mCi/mmol), [1-^{14}C] lauric acid (52.8 mCi/mmol), and [1-^{14}C] palmitic acid (57.0 mCi/mmol) were obtained from Amersham (Little Chalfont, United Kingdom), [1-^{14}C] sodium octanoate (55.0 mCi/mmol) from NEN (Boston, MA), and [1-^{14}C] sodium decanoate (10.6 mCi/mmol) from Sigma (St. Louis, MO). All the FA were obtained from Sigma. Thin-layer chromatography (TLC) plates were as follows: silica gel 60 (0.25 mm) from Merck (Darmstadt, Germany), KC18 Silica gel 60 (0.2 mm) from Whatman (Maidstone, England). All solvents were of analytical grade (Merck). Scintillation fluid-Ultima Gold was obtained from Packard (Meriden, CT). Polystyrene dishes were obtained from Corning (Corning, NY).

Tissue cultures. Human hepatoma HepG-2 cells were used in the experiments. HepG-2 cells were routinely grown in MEM supplemented with 10% FCS and 100 IU/L penicillin–streptomycin, in a 37ºC incubator with 5% CO_2. The experiments were done in 35-mm plastic dishes. When cells reached confluency, the growth medium was removed and replaced by 2 mL of fresh medium containing 0.1 mM of ^{14}C-labeled FA. All radiolabeled FA, except sodium decanoate, were mixed with the corresponding nonlabeled FA to a specific radioactivity of 10 mCi/mmole. These were dissolved in a small volume of ethanol (final concentration 0.05–0.1%), added to the growth medium, and incubated for 30 min at 37ºC (to ensure binding of the LCFA to albumin) prior to filtration through 0.22 µm sterile Millipore filters.

Under our experimental conditions, the cultures were incubated with the ^{14}C-FA for 1.5, 3, and 6 h. At the end of incubation, the media were removed, cells were washed once with 1% BSA in phosphate-buffered saline (PBS) to remove free FA and twice with PBS. All incubations were done in triplicate. Results are normalized for mg protein and represent the mean of three independent determinations. Variations did not exceed 5%.

Lipid extraction and analysis. Cells attached to the plates were extracted twice with hexane/isopropanol (3:2, vol/vol), first with 1.5 mL for 60 min and then with 1 mL for 15 min (17). The amount of the radioactivity in the extracts was determined using a Kontron-Betamatic counter (Zurich, Switzerland). Aliquots of the lipid extract were used for TLC. The hexane/isopropanol extracts were dried with a gentle stream of N_2 and the lipids dissolved in chloroform. Neutral lipids were separated on Silica gel G plates employing hexane/diethyl ether/methanol/acetic acid (90:20:3:2, by vol) as solvent. PL were separated using chloroform/methanol/acetic acid/water (100:20:12:5, by vol) as solvent.

TLC plates were dried and subsequently sprayed with 0.02% 2,7-dichlorofluorescein in ethanol. Lipid spots were detected under ultraviolet (UV) light, scraped into scintillation vials, and suspended in 3.5 mL of scintillation fluid for determination of radioactivity. The overlapping spot of diglycerides and cholesterol on the TLC plates was scraped and saponified with 0.5 M methanolic KOH for 30 min at 50°C. Samples were then acidified (with HCl 6 N) and extracted with hexane. Aliquots were placed on TLC plates as described above.

To determine the chain length of the FA which were incorporated into PL and TAG, the corresponding spots were collected and saponified with 0.5 M methanolic KOH for 30 min. FA were extracted with hexane after acidification. The solvent was removed with a gentle stream of N_2 at 37°C. FA were separated according to their chain length by reversed-phase TLC employing KC18 plates utilizing acetonitrile/methanol/water (6:3:1, by vol.) as solvent. Under our experimental conditions, 18:0 and 18:1 co-migrated with 16:0. This procedure was adapted from a method described for the separation of methyl esters on silanized silica gel G plates (18). FA were detected after spraying with Rhodamine 6G (0.1% in ethanol) under UV light. Spots were scraped directly into scintillation vials, and the radioactivity was counted immediately after addition of scintillation fluid.

Saturated and unsaturated fatty methyl esters were separated on $AgNO_3$-impregnated plates (19) employing hexane/diethyl ether (80:20, vol/vol) as solvent. FA were methylated with diazomethane according to Schlenk and Gellerman (20). The methyl esters were detected under UV light after spraying with 0.02% 2,7-dichlorofluorescein and scraped directly into scintillation vials, and the radioactivity was counted immediately after addition of scintillation fluid.

Methyl esters were separated by gas–liquid chromatography (GLC) on a 30-m PAG (polyalkylene glycol) column (0.25 µm film thickness; Supelco, Bellefonte, PA) at a temperature range of 185–220°C employing a Hewlett-Packard 5790A Gas Chromatograph (Palo Alto, CA) equipped with a flame-ionization detector. The relative composition of FA mixtures was calculated employing a Hewlett-Packard 3396 integrator. For quantitative analysis, heptadecanoic acid was added as an internal standard assuming that the response of the detector for all methyl fatty esters was identical.

To determine the secretion of radiolabeled lipids from the cells, medium was collected at the end of the incubation period. Media from three identical plates were pooled. Floating cells and debris were removed by centrifugation in the cold. The supernatant was extracted with chloroform/methanol according to Bligh and Dyer (21). Lipids were analyzed as described above.

Protein determination. Cells (after lipid extraction) were incubated overnight at room temperature with 2 mL 0.2 M NaOH to extract protein. Protein was determined according to the method of Markwell *et al.* (22). Each plate contained approximately 1.5 mg protein.

RESULTS

Incorporation of radioactive FA into cellular lipids by HepG-2 cells. To study the metabolism of FA of different chain lengths by HepG-2 cells, confluent cultures were incubated with 2 mL medium in the presence of 200 nmoles (0.1 mM) of [1-^{14}C]-labeled FA (C_2, C_8, C_{10}, C_{12}, and C_{16}) for 1.5, 3, and 6 h. Figure 1 shows the incorporation of these FA into cellular lipids. As can be seen from this figure, the incorporation of all the FA increased as a function of time. The incor-

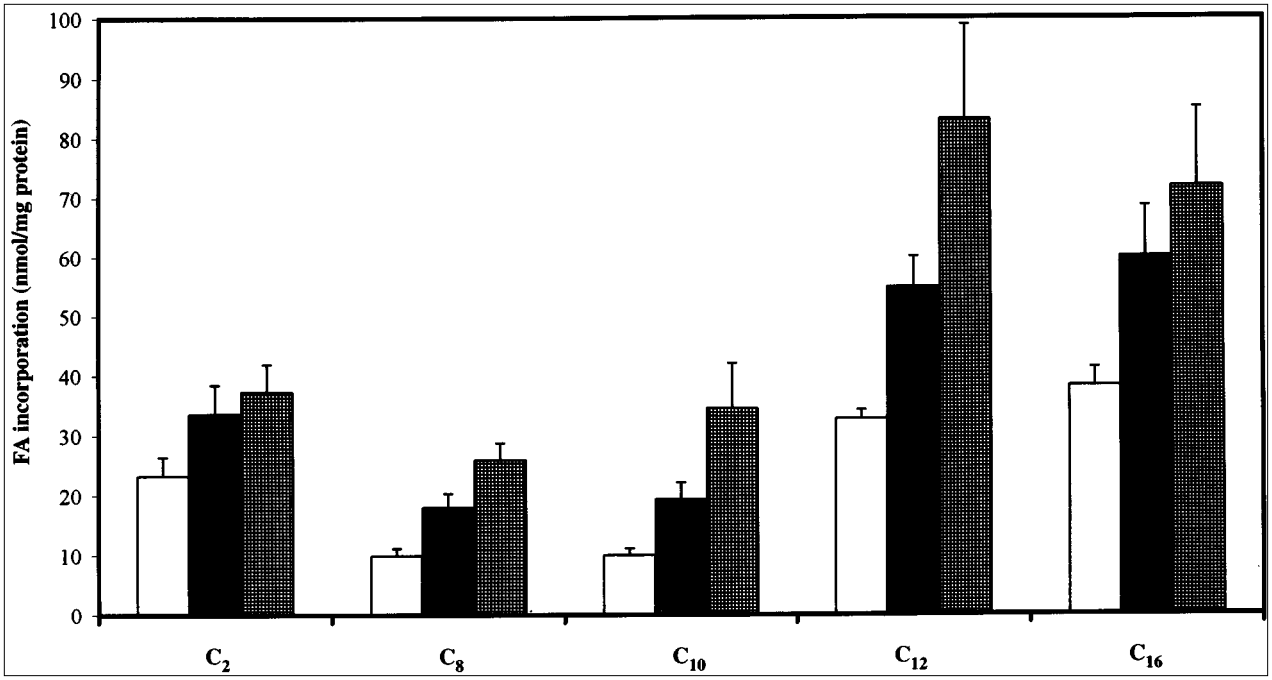

FIG. 1. Incorporation of different fatty acids (FA) into cellular lipids by HepG-2 cells. Confluent cultures were incubated for 1.5 [open box], 3 [diagonally striped box], and 6 h [hatched box], with 2 mL medium containing 200 nmoles (0.1 mM) of ^{14}C-labeled C_2, C_8, C_{10}, C_{12}, and C_{16}. The incorporation of ^{14}C into cellular lipids was determined as described in the Materials and Methods section. Each plate contained approximately 1.5 mg protein. Each point is the mean of three separate incubations ± SD.

poration of LCFA (C_{12} and C_{16}) was three to four times faster than that of MCFA (C_8 and C_{10}). After 1.5 h of incubation with LCFA, 17.5–20% of the added FA (35–40 nmoles per mg protein) were incorporated into cellular lipids as compared to 5% (10 nmoles per mg protein) of MCFA. C_2 showed a relatively high incorporation during the first 1.5 h, and the subsequent increase was smaller. Nevertheless the incorporation of C_2 was higher at all times than that of C_8 and C_{10}. The incorporation of C_{16} was rapid and amounted to approximately 50% of the applied radioactivity (70 nmoles/mg protein) after 3 h of incubation, thereafter, the rate of incorporation declined.

Distribution of labeled FA among cellular lipids. With all FA used, most of the radioactivity detected in the cells, at all times, was found in PL and TAG (Table 1). Only trace amounts of radioactivity were found in cholesterol, diacylglygerols, free FA, and cholesterol ester (not shown). As shown in Table 1, when cells were incubated for 1.5 h with C_2 or C_8, 85% of the radioactivity in cellular lipids was detected in PL. After 6 h, the fraction of radioactivity found in PL decreased (55% for C_2, 76% for C_8) while the radioactivity contained in TAG increased. The distribution of radioactivity in PL and TAG from C_{12} and C_{16} was approximately equal at all times. C_{10} yielded intermediate results. After 1.5 h of incubation, 78% was found in PL, decreasing to 61% after 6 h.

Separation of the PL by TLC showed that under our experimental conditions 70–72% of the radioactivity incorporated into PL was in phosphatidylcholine, 17–18% in phosphatidylethanolamine, and 10% migrated with lyso-PL (not shown).

In all cases, the amounts of the ^{14}C-labeled lipids synthesized in the cells and released into the medium did not exceed 1 to 2%. Similar results were reported by other investigators (23,24).

FA composition of newly synthesized PL and TAG. Following separation of the radiolabeled cellular PL and TAG, their FA were isolated and separated according to chain length as described in the Materials and Methods section. C_2 and C_8 were used exclusively for the synthesis of LCFA (not shown). With C_{16} as precursor, all the radiolabeled FA found in both TAG and PL, after 1.5, 3, or 6 h of incubation, were LCFA (not shown). ^{14}C-C_{12} was largely incorporated unchanged. As can be seen from Figure 2, 55 to 65% of the FA found in PL, and about 87% of the labeled FA in TAG were ^{14}C-C_{12}. ^{14}C-C_{10} yielded intermediate results. A significant fraction of the C_{10} taken up by the cells was recovered as C_{10} in TAG and PL. As can be seen from Figure 2, approximately 10% of the ^{14}C-FA detected in PL were C_{10} (the rest being LCFA). In TAG, up to 40% of the radioactive FA were ^{14}C-C_{10}.

Separation of the radiolabeled FA according to the degree of unsaturation showed that a considerable fraction of the ^{14}C-FA were monounsaturated (Table 2). Only negligible amounts of radioactive polyunsaturated FA were detected (not shown). The fraction of monounsaturated FA in both PL and TAG as studied after various periods of incubations de-

TABLE 1
Incorporation of Different Fatty Acids (FA) into Cellular Phospholipids (PL) and Triacylglycerols (TAG)[a]

FA added	Radioactivity incorporated (%)					
	1.5 h		3 h		6 h	
	TAG	PL	TAG	PL	TAG	PL
C_2	11.55 ± 2.00	85.10 ± 2.20	15.80 ± 2.99	80.41 ± 2.95	19.87 ± 3.11	75.49 ± 1.32
C_8	10.81 ± 1.51	85.45 ± 1.37	13.54 ± 2.12	82.29 ± 2.65	19.04 ± 0.51	76.48 ± 0.58
C_{10}	18.17 ± 2.3	78.12 ± 2.43	25.43 ± 1.31	71.61 ± 1.10	33.97 ± 4.15	61.81 ± 4.20
C_{12}	44.21 ± 4.98	51.43 ± 4.76	47.40 ± 4.59	47.53 ± 4.50	52.66 ± 2.86	43.56 ± 2.86
C_{16}	36.11 ± 1.07	59.26 ± 0.98	40.97 ± 1.34	55.10 ± 1.10	42.32 ± 3.17	54.45 ± 3.42

[a]Cells were incubated with ^{14}C-labeled FA as described in the legend to Figure 1. Aliquots of the lipid extracts were subjected to thin-layer chromatography, and separated with hexane/diethyl ether/methanol/water (90:20:3:2, by vol). Lipid spots were detected with 2,7-dichlorofluorescein, scraped directly into scintillation vials, and the radioactivity determined as described in the Materials and Methods section. Each result is a mean (± SD) of three separate determinations.

pended on the chain length of the FA added to the incubation medium. The fraction of monounsaturated FA was highest (25–30%) after incubation with C_2 and did not change throughout the incubation period. In the presence of ^{14}C-labeled longer-chain FA, the percentage of ^{14}C monounsaturated FA was smaller. After 1.5 h of incubation with C_8 and C_{10} or C_{12} and C_{16}, approximately 15% and 10–12%, respectively, were detected in the PL. These amounts doubled after 6 h and reached 27% for C_8 and C_{10} and 20–24% for C_{12} and C_{16}. The percentage of ^{14}C-monounsaturated FA in TAG did not change markedly during the incubation period and remained at 25–27% for C_8 and C_{10} and 13–17% for C_{12} and C_{16}. The synthesis of more ^{14}C-monounsaturated FA in the presence of C_2 suggests that extensive desaturation occurs mainly during *de novo* synthesis of LCFA.

In an attempt to detect changes in the overall FA composition of cellular PL and TAG due to incubation with externally added FA, the composition of these lipids after 3 h of incubation was resolved by GLC as described in the Materials and Methods section. An important finding of this analysis, as shown in Table 3, is that following incubation with C_{12} a significant fraction of the FA in PL (1.9%) and TAG (9.8%) was C_{12}. A similar but much smaller effect was found after incubation with C_{10}. Neither C_{12} nor C_{10} was detected in cells following incubation in any other medium. To compensate for the incorporation of C_{12} into TAG, a significant reduction occurred in 18:1 (from 46.4 to 37.7%). A smaller reduction was noted in the percentage of 18:0. C_{10} caused much smaller changes in 18:1. Likewise, when cells were incubated in the presence of C_{16}, the percentage of C_{16} in cellular TAG in-

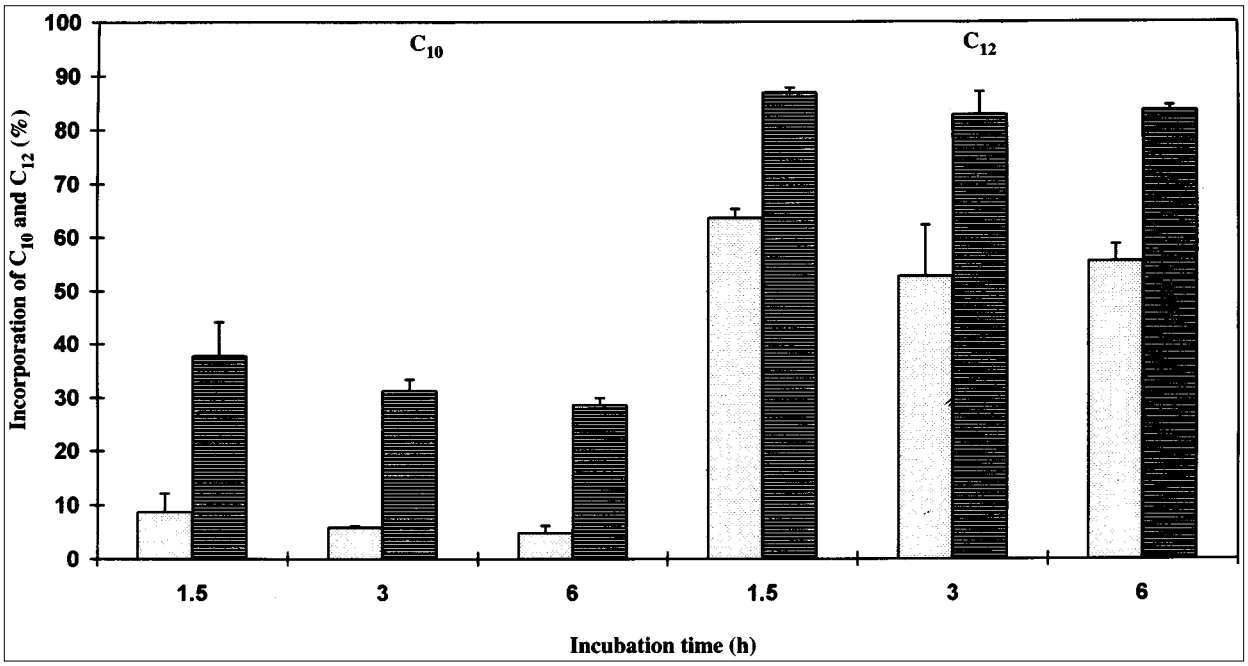

FIG. 2. Recovery of C_{10} and C_{12} in cellular phospholipids and triacylglycerols. Cells were incubated as described in the legend to Figure 1. Lipid extracts of three incubations were pooled and separated by thin-layer chromatography (TLC) as described in Table 1; phospholipids [dotted box] and triacylglycerols [vertically striped box] were scraped off the plate and subjected to alkaline hydrolysis. Fatty acids were recovered and separated by reverse-phase TLC as described in the Materials and Methods section. Each point is the mean of three separate incubations ± SD. C_2, C_8 and C_{16} yielded only long chain fatty acid.

TABLE 2
Occurrence of Radiolabeled Monounsaturated FA[a]

FA added	Radiolabled monounsaturated FA (%)					
	1.5 h		3 h		6 h	
	TAG	PL	TAG	PL	TAG	PL
C_2	33.64	25.64	31.30	26.33	29.01	28.12
C_8	25.73	14.85	21.50	22.59	26.31	27.30
C_{10}	24.68	15.92	27.03	21.79	27.04	27.21
C_{12}	17.70	10.22	13.32	15.88	17.96	20.14
C_{16}	7.84	12.39	10.93	18.09	11.74	24.40

[a]Cells were incubated with ^{14}C-labeled FA as described in the legend to Figure 1. The methyl fatty esters obtained from PL and TAG were separated on $AgNO_3$-impregnated plates as described in the Materials and Methods section. Spots corresponding to saturated and monounsaturated FA were scraped directly into scintillation vials and radioactivity determined immediately as described in the Materials and Methods section. See Table 1 for abbreviations.

TABLE 3
FA Composition of Cellular PL and TAG[a]

FA		10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	20:3	20:4	20:5	22:6
C_8	TAG	—	—	2.5	24.9	7.3	7.1	46.5	0.6				
	PL	—	—	1.7	21.3	10.0	11.0	33.0	3.3	4.5	5.0	0.5	3.6
C_{10}	TAG	0.4	—	3.0	26.5	8.0	6.7	44.7	1.4				
	PL	0.1	—	1.7	21.5	9.1	10.6	32.3	3.1	4.2	4.3	0.4	2.6
C_{12}	TAG	—	9.8	3.6	26.0	7.4	5.6	37.7	1.2	4.3	1.9		
	PL	—	1.9	2.3	22.2	9.2	10.0	30.6	3.0	4.1	4.6	0.6	3.4
C_2[b]	TAG	—	—	1.4	18.7	3.1	9.0	57.1	1.0	4.3	1.9		
	PL	—	—	1.2	21.5	4.1	10.0	36.7	3.9	0.6	4.6	5.7	0.5
C_{16}[b]	TAG	—	—	2.2	30.3	8.0	5.5	42.7	1.5	4.0	1.1	0.7	2.9
	PL	—	—	0.9	20.2	8.0	11.8	30.5	1.5	1.7	8.5	5.4	1.7

[a]Cells were incubated with ^{14}C-labeled FA for 3 h as described in the legend to Figure 1. Extracts of triplicates were pooled, PL and TAG were separated by thin-layer chromatography as described in the legend to Table 1. The spots corresponding to PL and TAG were collected and subjected to alkaline hydrolysis. FA were recovered after acidification, methylated with diazomethane, and separated by gas–liquid chromatography as described in the Materials and Methods section.
[b]Cells were grown on a different batch of fetal calf serum. See Table 1 for abbreviations.

creased while 18:1 decreased. Cells grown in the presence of C_2 contained 18.7% 16:0 and 57.1% 18:1 in cellular TAG, in contrast to cells grown in the presence of C_{16} which contained 30.3% 16:0 and 42.7% 18:1. The changes in the FA composition of PL were much smaller. However since the cells contained approximately four times more PL than TAG these differences can be easily explained. We have noted in several experiments (not shown) that the FA composition of the FCS markedly affects the FA composition of the HepG-2 cells. A new batch of FCS was used for the incubation of cells with ^{14}C-C_2 and ^{14}C-C_{16}. This can explain the relatively high percentage of 18:1 and low percentage of 16:0 found in these cells when incubated in the presence of C_2. The GLC results as shown in Table 3 indicate the occurrence of 6.4 and 8.5 nmoles/mg protein of C_{12} in PL and TAG, respectively. These results are 40–50% of the accumulation expected from ^{14}C-C_{12} incorporation experiments, probably indicating losses of methylated C_{12} (which is much more volatile than the free acid) during preparation of samples for GLC analysis.

DISCUSSION

In an attempt to explain the results of our recent clinical studies (7) in which we have observed marked changes in the composition of plasma and biliary lipids following the infusion of MCT/LCT, a study of the metabolism of FA of different chain lengths by isolated liver cells was undertaken. Our experimental approach involved incubation of HepG-2 with ^{14}C-FA of different chain length for up to 6 h and subsequent analysis of the incorporation of these FA or their metabolites into cellular PL and TAG. These studies showed that the metabolism of C_{10} was unique:

C_2 and C_8 were used exclusively for the synthesis of saturated and monounsaturated LCFA, suggesting that C_8 was oxidized to C_2 units prior to being used for FA synthesis, as has been shown previously (25). The newly synthesized ^{14}C-FA were incorporated preferentially (85%) into PL (Table 1).

C_{12} and C_{16} were incorporated, mostly unchanged, in approximately equal amounts into cellular PL and TAG (Figs. 1 and 2; Table 1). Recently it was shown by Gibbons *et al.* (26) that cultured HepG-2 cells readily incorporated ^{14}C-oleate into cellular lipids, approximately 90% into TAG and 10% into PL.

The fate of C_{10} was more complex; it was different from that of both LCFA and SCFA and represented an intermediate between these pathways. ^{14}C-C_{10} yielded ^{14}C-LCFA, either by elongation of internalized ^{14}C-C_{10} or by *de novo* synthesis after oxidation to C_2 units, but considerable amounts of un-

changed ^{14}C-C_{10} were detected in PL—10%—and TAG—40% (Table 1, Fig. 2).

Separation by GLC of the FA obtained from PL and TAG showed the accumulation of C_{12} and to a lesser extent C_{10} only when HepG-2 cells were incubated in the presence of the respective FA. These results demonstrate that in addition to the mammary gland, liver cells can incorporate C_{10} and C_{12} into cellular lipids. Woollett *et al.* (5) did not find C_{10} in cellular liver lipids. In our studies with HepG-2 cells, the concentration of preformed lipids was much smaller than in intact liver, and hence the dilution of externally added FA was much smaller which allows their detection by GLC.

The mechanism of FA uptake by hepatic parenchymal cells is not well understood (27), and the chain length dependence of this process has not been systematically investigated thus far. Two factors that are likely to play an important role in the observed chain length-dependence of FA uptake are the binding to albumin (in the incubation medium) and to the intracellular fatty acid-binding proteins. Both these proteins have much higher binding affinities for LCFA than for SCFA and MCFA (27–29). Recent studies have indicated that serum albumin has a direct role in FA uptake, rather than merely a passive role as a carrier of LCFA, through interaction with the cell surface (15). These observations may explain the faster incorporation of C_{12} and C_{16} in comparison to C_2 and C_8 and C_{10}. An alternative mechanism for the uptake of FA into hepatocytes is supported by the recent demonstration of an FA-transporter in hepatocyte membranes (30). The affinity of this transporter to SCFA and MCFA has yet to be tested.

In addition to the dependence of internalization rates on FA chain length, differences in the intracellular utilization also occur. When taken up by hepatocytes, a large fraction of both SCFA and MCFA is oxidized by the mitochondria to acetyl CoA (11,31).

Our results have several important implications with respect to human nutrition. Prolonged infusion of MCT (rich in C_8, C_{10}, and C_{12}) can be expected to result in enrichment of plasma lipoproteins with C_{10} and C_{12} TAG, as has in fact been shown (32). The possible enrichment of hepatic PL with C_{10} and C_{12} may affect membrane fluidity and cell function. Furthermore, changes in biliary PL composition may occur which will affect cholesterol solubility and gallstone formation (33). Recently we have observed that upon perfusion of rat liver with radiolabeled FA of different chain lengths, significant amounts of ^{14}C-C_{10} and ^{14}C-C_{12} became incorporated into biliary PL (Pakula, Ronit, and Alisa Tietz, unpublished results).

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Effect of Docosahexaenoic Acid on Mouse Mitochondrial Membrane Properties

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ABSTRACT: Long-chain polyunsaturated (n-3) fatty acids have been proposed to be involved in a wide variety of biological activities. In this study, mitochondrial docosahexaenoic acid (DHA) levels were increased by either dietary manipulation or by fusing the mitochondria with phospholipid vesicles made from 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (18:0/22:6 PC). The fused mitochondria exhibited a DHA-induced decrease in respiratory control index (RCI) and membrane potential and an increase in proton movement. The modified mitochondria also demonstrated an increase in fluidity (as detected by 1,6-diphenyl-1,3,5-hexatriene anisotropy) and changes in membrane structure detected by the fluorescence probes MC540 and pyrene decanoate. Proton movement in lipid vesicles made from mitochondrial lipid extracts was shown to be enhanced by incorporated 18:0/22:6 PC. Mitochondria were isolated from young (5-mon) and old (24-mon) mice which were maintained on either a diet rich in saturated fats (hydrogenated coconut oil) or rich in n-3 polyunsaturated fats (menhaden oil). Mitochondrial bioenergetic function was followed by RCI, state 3 respiration, ATP level, and phosphate uptake. In addition, lipid composition, phospholipid area/molecule, and extent of lipid peroxidation were also determined. Decreases in RCI for the menhaden oil diet-modified mitochondria paralleled those in which DHA levels were enhanced by fusion with phospholipid vesicles. RCI reductions are attributed to DHA-induced increases in H^+ movement, producing diminished mitochondrial membrane potentials. One purpose of this project was to determine if the deleterious effects of aging on mitochondrial bioenergetic function could be reversed by addition of n-3 fatty acids. The experiments reported here indicate that incorporation of long-chain polyunsaturated n-3 fatty acids into mitochondrial membranes does not appear likely to reverse the effects of age on mitochondrial function.

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The n-3 fatty acids have recently received a great deal of attention due to their potential role in treating a number of human afflictions including cancer, heart disease, and arthritis (1). While the molecular mode of action of these fatty acids remains a mystery, it has been shown that they are efficiently taken up into rapidly growing cells where they may alter membrane events (2,3). Because membrane structure is known to reflect its fatty acid composition (chain length and number of unsaturations) (4), it is not surprising that long-chain polyunsaturated n-3 fatty acids, particularly docosahexaenoic acid (DHA, $22:6^{\Delta 4,7,10,13,16,19}$), have been shown to substantially influence membrane structure/function (1,5,6).

A variety of reports have indicated that the mode of action of DHA may involve perturbations of the plasma membrane. DHA incorporation into phospholipid vesicles as either free acid or 18:0/22:6 phosphatidylcholine (PC) has resulted in large increases in membrane permeability. T27A tumor cells (6) and JURKAT leukemia cells (7) were shown by ^{51}Cr release to become leaky in the presence of sufficient free DHA (7). Permeability was also augmented after fusing the T27A cells with lipid vesicles made from 18:0/22:6 PC (6). Burns and Spector (8) have shown that ascites lymphoblastic leukemia cells became permeable to the anticancer drugs mitoxentrone and doxorubicin upon incorporation of DHA. Membranes of the rod outer segment (ROS), known to be highly enriched in DHA (9), are very permeable to K^+ (10). While all of these reports indicate that DHA increases membrane permeability, none link the increase with a change in a specific physiological process.

Here we investigate the effect of DHA on H^+ permeability (movement) across the mitochondrial inner membrane. Mitochondria are an excellent system to study permeability because DHA membrane levels are easily manipulated, and a correlation between permeability (to H^+) and a biological process (bioenergetics) is readily monitored. Mitochondrial DHA levels are controlled by either fusing isolated mitochon-

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Abbreviations: BCECF, 2′, 7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; BCECF-AM, 2′, 7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; DHA, docosahexaenoic acid; $DiSC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide; DPH, 1,6-diphenyl-1,3,5-hexatriene; E/M, exciner/monomer; EPA, eicosapentaenoic acid; HCO, hydrogenated coconut oil; MC540, merocyanine 540; MO, menhaden oil; O-HCO, old mice on the hydrogenated coconut oil diet; O-MO, old mice on the menhaden oil diet; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RCI, respiratory control index; SUV, small unilamellar vesicles; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; Y-HCO, young mice on the hydrogenated coconut oil diet; Y-MO, young mice on the menhaden oil diet; 18:0/18:1 PC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 18:0/22:6 PC, 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine.

dria with phospholipid vesicles composed of 18:0/22:6 PC or by feeding mice a diet rich in DHA (menhaden oil). If DHA increases mitochondrial membrane permeability, as it does for lipid vesicles, tumor cells and the ROS, we would anticipate finding substantial DHA-induced alterations in proton gradient-dependent bioenergetic events.

EXPERIMENTAL PROCEDURES

Materials. The fluorescent probes BCECF, BCECF-AM, $DiSC_3(5)$, DPH, MC540, pyrene decanoate (1-pyrene hexadecanoate), and TMA-DPH were purchased from Molecular Probes (Eugene, OR). The phospholipids 18:0/18:1 PC and 18:0/22:6 PC were from Avanti Polar Lipids (Alabaster, AL). BF_3/methanol was from Sigma Chemical Co. (St. Louis, MO).

Mice and diets. Experiments in which mitochondria were fused with lipid vesicles containing DHA were performed with BALB/c male mice. Four-week-old mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and fed a modified AIN-76 diet (referred to here as the HCO diet) containing 2 g of vitamin E per kg diet, 0.5% corn oil for essential fatty acids, and an additional 10% fat as hydrogenated coconut oil (HCO) (ICN, Cleveland, OH) for three weeks before being euthanized.

For the diet experiments involving young and old mice, 2-mon-old (young) and 21-mon-old (old) CBA/Ca female mice were purchased from The National Institute of Aging Colonies, Charles River Laboratories (Wilmington, MA) and enrolled for 7 wk, 5 d on the HCO diet. The HCO-fed mice were then either switched to a diet in which the HCO was replaced by 10% menhaden oil (MO) (the MO was supplied by Zapata-Haynie Corp., Reedville, VA) or maintained on the 10% HCO. After 3 wk on the experimental diets, the mice were euthanized and their livers extracted for the mitochondrial studies. At 21 mon old, CBA female mice are nearing the end of their lifespan and so indeed are "old." The average lifespan reported by the Jackson Laboratory for virgin CBA/J females is about 18 mon, and about 22 mon for several common inbred mouse strains. The diet used here is based on a previous study from our laboratory (11). The function of the initial HCO diet is to reduce the membrane DHA content accumulated during a lifetime on "chow" which contains fish meal. The subsequent 3-wk diet is average for liver modification studies, which have regimens ranging from 10 d to 5 wk (12–14). Due to the age of the test animals, more prolonged feeding experiments were not possible. The saturated fat HCO diet is used as a model of the typical Western diet and provides a sharp contrast to the n-3-rich MO diet.

Mitochondria. Mouse liver mitochondria were isolated by the method of Johnson and Lardy (15). The isolation buffer contained 250 mM sucrose, 20 mM Tris-HCl, 0.5 mM EDTA, and 0.1% bovine serum albumin (pH 7.4). Mitochondrial protein was determined by the method of Bradford (16). Isolated mitochondria were always kept on ice in the dark until they were tested for respiratory control index (RCI), state 3 respiration, ATP level, phosphate uptake, lipid composition, lipid area/molecule, extent of lipid peroxidation, proton movement (permeability), fluidity, membrane structure, and membrane potential.

Lipid vesicle–mitochondria fusion studies. Small unilamellar vesicles (SUV) were made by sonication (2 min at level 5 on a Heat Systems-Ultrasonics Model W-380; Plainview, NY) of either 18:0/18:1 PC or 18:0/22:6 PC multilamellar vesicles at 5 mg lipid/mL of mitochondrial isolation buffer. Mitochondria (5 mg protein/mL) were gently mixed on ice for 35 min with SUV to give a final PC concentration of 0, 0.5, 1.0, 1.5, or 2.0 mg/mL. Fused mitochondria were separated from nonfused SUV by centrifugation at 5,000 rpm. After a wash in the isolation buffer, the pelleted mitochondria were resuspended in the same buffer to a final protein concentration of 3 mg/mL.

Fatty acid compositions. Total mitochondrial lipids were extracted with chloroform/methanol 2:1 (vol/vol) (17) and filtered on Millipore filters (Milford, MA). Phospholipid content was determined as outlined by Ames (18). This same extract was also used to determine the fatty acid profiles (19). The phospholipids were first saponified in 0.5 N NaOH by refluxing the solution for 1 h in the presence of 0.2% of the antioxidant *tert*-butyl hydroquinone. Methyl esters were made by addition of 14% BF_3/methanol and additional refluxing for 30 min. The methyl esters were extracted into hexane three times and evaporated to a small volume and then injected into a Shimadzu G17A Gas Chromatograph (Tokyo, Japan) equipped with an autosampler. Retention times were measured on a stabilwax capillary column (Restek, Bellefonte, PA) and were compared to methyl ester standards purchased from Nu-Chek-Prep (Elysian, MN). Lipid peroxidation was followed by fluorescent detection of malondialdehyde with 2-thiobarbituric acid. The method is that outlined by Tatum *et al.* (20), with the following modification: Total mitochondrial lipid was extracted by $CHCl_3$/MeOH (2:1) and the phases separated upon the addition of aqueous 0.5 N NaCl. The protein-free lipid extract was used for the peroxidation assays.

Mitochondrial bioenergetics. Mitochondrial oxygen consumption was followed by the method of Estabook (21) as modified by Gazzotti *et al.* (22) using polarography with Clark-type electrodes. Oxygen consumption was measured on a YSI 5300 Biological Oxygen Monitor System (Yellow Springs Instruments, Yellow Springs, OH). Temperature was controlled at 30°C by a Haake Circulating Water Bath (Karlsruhe, Germany). High-sensitivity membranes were used for all measurements. Mitochondria (approximately 2–5 mg of protein) were added to 3.0 mL of the oxygen consumption buffer (5 mM KH_2PO_4, 120 mM KCl, 20 mM Tris, pH 7.4). Substrate (60 μL of a 250 mM glutamate solution) was injected into the sealed mitochondrial chamber, and the respiration rate was followed for 2 min. To initiate state 3 respiration, 30 μL of a 50 mM solution of ADP was then injected into the suspension. Respiratory control indices (RCI) were calculated by measuring the state 3 to state 4 respiration rates.

ATP levels were estimated by using a Sigma Diagnostics kit (Cat.# 366-UV) (St. Louis, MO) and are expressed as µg ATP/mg protein.

Mitochondrial swelling. Alterations in the activity of the mitochondrial phosphate carrier were followed by osmotic volume changes (swelling) (23). This assay was selected because it is known to be particularly affected by age and membrane lipid composition (23). Mitochondrial swelling (30°C) in 125 mM $(NH_4)_2PO_4$, 5 mM HEPES, 0.5 mM EDTA, pH 7.4 was followed by light scattering at 540 nm on a Perkin-Elmer Lambda 4C UV/Vis Spectrophotometer interfaced to a Perkin-Elmer 7700 Professional Computer and run by PECUV software (Palo Alto, CA). Absorbance (A) changes were monitored for the first 30 s and the initial swelling velocity calculated and expressed as d(1/A)/dt percentage (24).

Mean molecular area. The chloroform/methanol 2:1 (vol/vol) (13) extracts, dried under nitrogen and vacuum, were employed for the mean molecular area determinations. Stock solutions of 1 mg/mL were made in triply distilled benzene and were spread over a subphase of 10 mM Tris, 10 mM sodium acetate, pH 7.4 in deionized, glass distilled, Milli-Q water (Millipore Corp., Bedford, MA). Benzene was allowed to evaporate for 3 min before the compressions were started. Mean molecular area was calculated using a KSV Minitrough (KSV Instruments, Helsinki, Finland) interfaced to an Epson Equity 2+ computer and run by LB5000 software. Monolayers were compressed at 1 mN/min with a final target pressure of 20 mN/M. All measurements were made at 30°C, and the results are the average of three separate compressions.

Proton movement. Proton movement was determined using the fluorescent probe BCECF-AM (25). Mitochondria were incubated for 20 min at 27°C with BCECF-AM at 5 µg probe/2 mg mitochondrial protein. A pH gradient was established across the mitochondrial inner membrane by titrating a small volume of 6 N HCl into three mL of the mitochondrial suspension (5 mg protein/mL) in the initial isolation buffer. Fluorescence was monitored for one minute (27°C) on a Perkin-Elmer MPF-66 fluorimeter (Norwalk, CT). Excitation and emission wavelengths were 500 nm and 530 nm, respectively. The initial pH was 7.4 and the final pH between 4.9 and 5.2. Proton movement was also determined for lipid vesicles made from the chloroform/methanol 2:1 (vol/vol) (17) extract augmented with increasing levels of 18:0/22:6 phosphatidylcholine (PC).

Mitochondrial membrane potentials. Relative mitochondrial membrane potentials were determined using the fluorescent probe $DiSC_3(5)$ (26). To 3.0 mL of a mitochondrial suspension containing 1 mg protein/mL in isolation buffer, 10 µL of $DiSC_3(5)$ (0.33 mg/mL in DMSO) was added. Membrane potential was established by addition of 12 µL of 500 mM succinate. Fluorescence (excitation at 620 nm and emission at 670 nm) was monitored for 2 min.

Fluorescent membrane probes. Docosahexaenoic acid (DHA) effect on membrane fluidity was followed with the fluorescence probes DPH and TMA-DPH while qualitative DHA-induced changes in membrane structure were probed with MC540 and pyrene decanoate. All measurements were made on a Perkin-Elmer MPF-66 fluorimeter, temperature controlled to 27 ± 0.1°C. Very dilute mitochondrial suspensions were employed to diminish light scattering, and samples were always kept in the dark to prevent photobleaching.

DPH and TMA-DPH. The fluorescent probe (in tetrahydrofuran carrying solvent) was incubated with 0.5 mg mitochondrial protein/mL for 30 min at 27°C. Probe to lipid molar ratio was 1:200. Anisotropies were measured upon exciting the samples at 358 nm with emission at 426 nm.

Pyrene decanoate. Pyrene decanoate (1-pyrene hexadecanoate in tetrahydrofuran/DMSO, 1:1) was incubated with 0.5 mg mitochondrial protein/mL for 30 min at 27°C. Probe/lipid molar ratio was 1:100. Excitation was at 340 nm, and the emission spectra followed from 360–560 nm.

MC540. MC540 was added to the mitochondrial suspensions from an aqueous stock solution (25 µM in 0.01M Na acetate, pH 7.4) to give a 1:150 probe to lipid molar ratio. MC540 was excited at 540 nm, and the emission spectra recorded from 560 to 650 nm.

RESULTS

DHA was incorporated into mitochondria by two methods. Either isolated mitochondria were fused with 18:0:22:6 PC vesicles or mice were enrolled on a diet rich in DHA [menhaden oil (MO) diet].

Mitochondrial fusion with 18:0:22:6 PC vesicles. Fatty acid analysis of mitochondria incubated with 18:0/22:6 PC SUV, revealed increasing DHA levels with increasing lipid concentrations of SUV (Fig. 1). DHA levels in the unmodified mitochondria were normally quite low, less than 1% of the total fatty acids present. The other predominant fatty acids

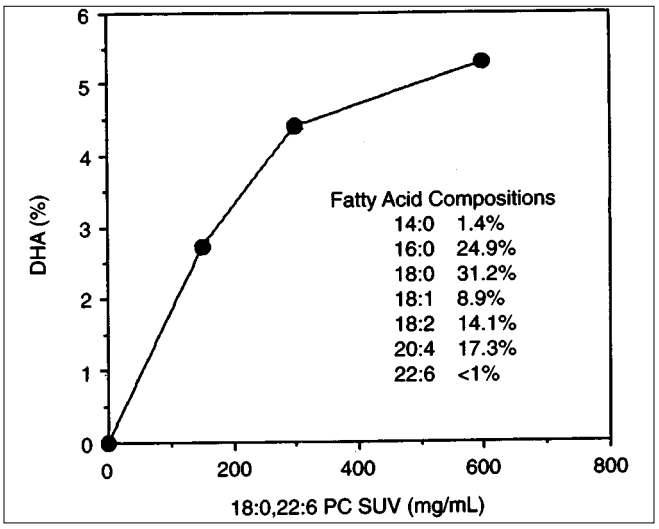

FIG. 1. Incorporation of docosahexaenoic acid (DHA) (expressed as the mol%) into mitochondria by fusion with increasing amounts of 18:0/22:6 PC SUV in the bathing solution. A profile of the major mitochondrial fatty acids is reported in the insert.

found in the BALB/c mouse mitochondria are listed in the insert of Figure 1. The highest level of DHA we obtained after fusion was about 7% for mice that were maintained on a well-defined AIN-76 diet. Care must be taken with conventional rodent diets since they contain fish meal, an excellent source of DHA. We found mice on conventional diets often contained more than 10% DHA in their mitochondria, even when the diet was not supplemented with an additional n-3 source. Variations in DHA levels, commonly reported for mouse mitochondria, likely reflect prior consumption of conventional diets.

In our experiments, we found decreases in mitochondrial bioenergetic functions associated with increasing DHA levels. Tightly coupled mitochondria isolated from 2-mon-old BALB/c mice were uncoupled (expressed as a decrease in RCI) upon the accumulation of small amounts of 18:0/22:6 PC (but not 18:0/18:1 PC) in the mitochondria (Fig. 2). For the 18:0/22:6 PC-augmented mitochondria, there was about a 30% decrease in the RCI with 5% incorporation of DHA. Associated with this was a 60% increase in H+ movement and a 30% decrease in membrane potential (Fig. 3). In contrast, 18:0/18:1 PC augmentation had little effect on the measured RCI (Fig. 2).

Fluorescent probes were used to detect DHA-induced changes in physical properties of the lipid bilayer component

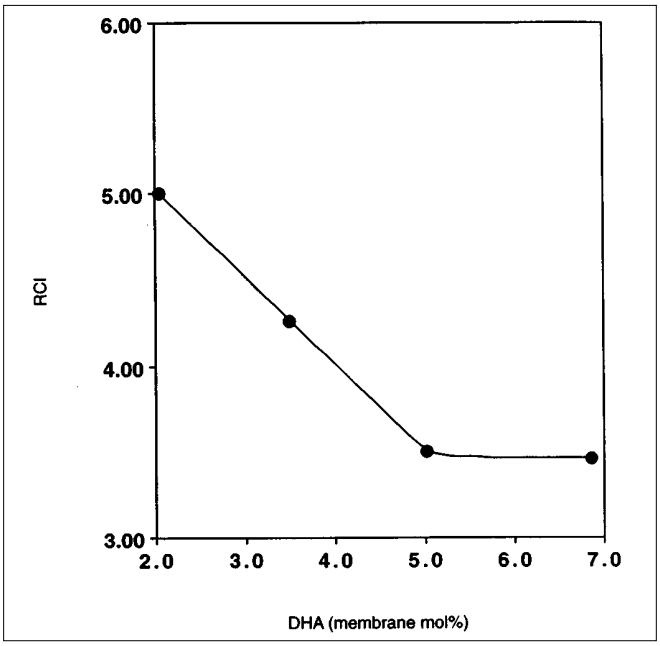

FIG. 2. Respiratory control indices (RCI) for mitochondria, isolated from young mice, that were fused with 18:0/22:6 phosphatidylcholine (PC) vesicles. The incorporation of DHA (22:6) increased from 2.0% for the unfused control to 6.9% for the mitochondria fused with 2.0 mg/mL of 18:0/22:6 PC. For all experiments, unfused mitochondria had less than 0.5% DHA. Controls were performed by fusing 18:0/18:1 PC with the mitochondria at the same concentrations used for the 18:0/22:6 PC fusions. This control lipid had no deleterious effect on RCI values which remained between 5.3 to 5.6. Results are the mean of three determinations. See Figure 1 for abbreviation.

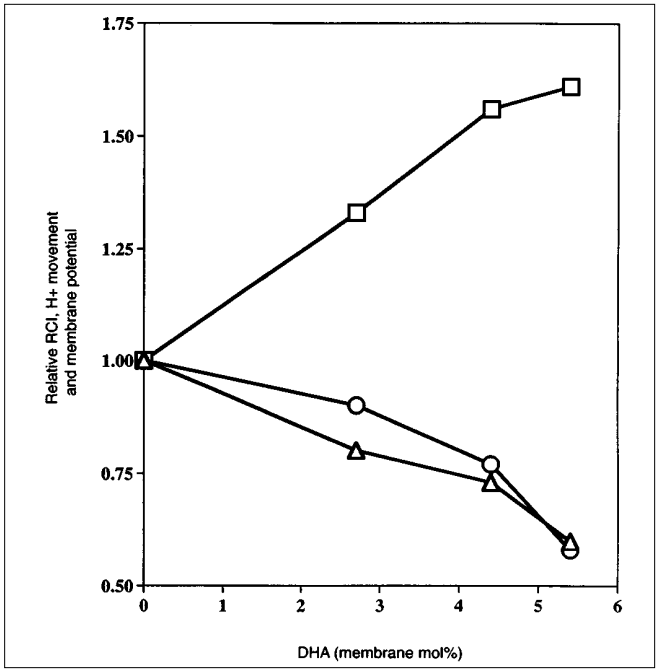

FIG. 3. Relative RCI (-△-), H+ movement (-□-) and membrane potential (-○-) for mitochondria that were fused with 18:0/22:6 PC. RCI were determined by polarography, proton permeability by use of the fluorescent probe 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester, and membrane potentials by the fluorescent probe $DiSC_3(5)$. Values are normalized to the unfused controls. Experimental details are outlined in the Materials and Methods section. See Figures 1 and 2 for abbreviations.

of the mitochondrial membrane. A small DHA-dependent decrease in anisotropy (increase in fluidity) was noted for the membrane interior probe DPH while the companion probe, TMA-DPH, did not detect any DHA-induced change at the aqueous interface (results not shown). MC540 and pyrene decanoate also demonstrated a DHA-induced change in membrane structure. The fluorescent membrane probe MC540 (merocyanine 540) has been used extensively to determine relative phospholipid packing on the outer surface of membranes (27). A fluorescence increase at 590 nm, as reported in Figure 4, can be interpreted as resulting from a decrease in lipid packing on the membrane outer leaflet. The increase in 590 nm fluorescence is linearly proportional to the level of incorporated DHA (insert, Fig. 4). The fluorescent probe pyrene decanoate has been extensively used to monitor membrane viscosity. Two fluorescence peaks are of particular interest. The peak at 480 nm is due to excimers and is enhanced in liquid crystalline state, while the peak at 398 nm is due to monomers and reflects gel state in membranes. Therefore the DHA-dependent increase in fluorescence intensity at 480 nm (Fig. 5) indicates that the bilayer is exhibiting a more fluid environment in the liquid crystalline state. The small change at 398 nm indicates that DHA has less effect on the gel state component. In the insert of Figure 5, the E/M (excimer/monomer) ratio is plotted against the amount of DHA incorporated

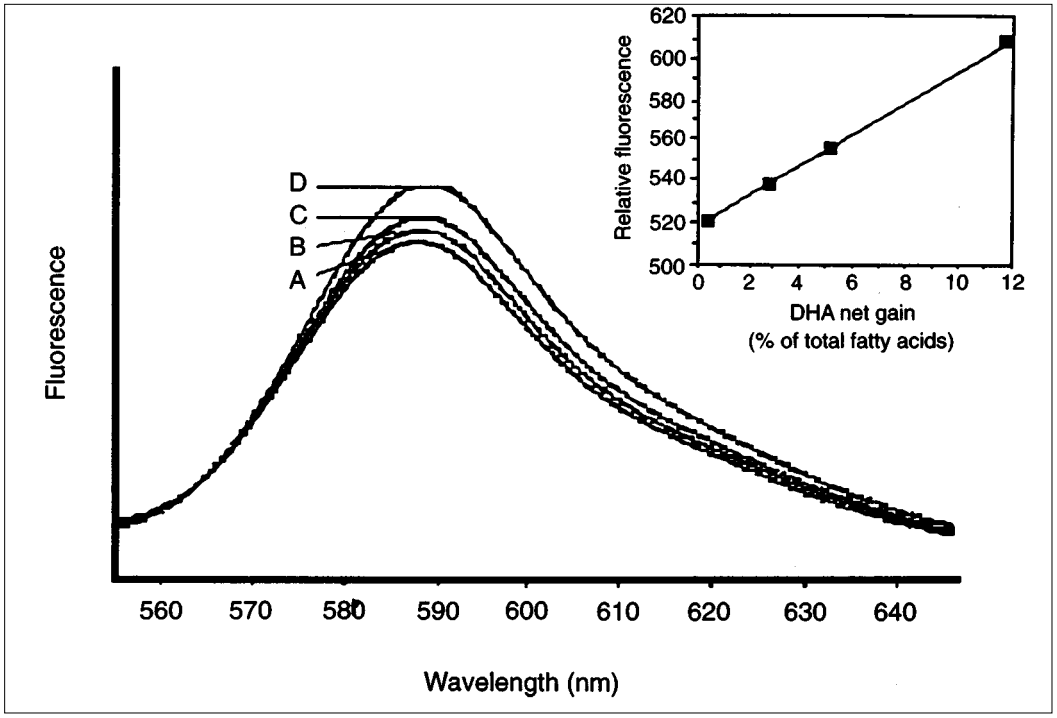

FIG. 4. Mitochondrial membrane structure (monitored by the surface probe MC540) as a function of increasing amounts of incorporated DHA. Insert compares the relative fluorescence at 590 nm as a function of the amount of membrane-incorporated DHA. See Figure 1 for abbreviation.

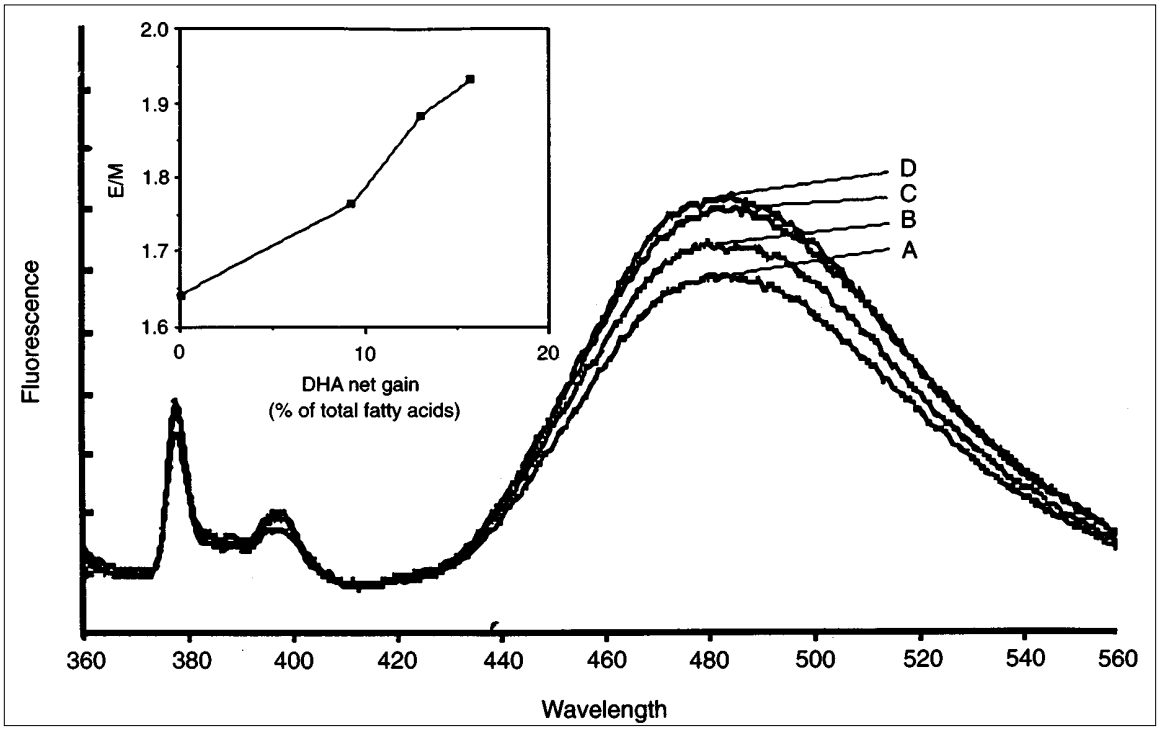

FIG. 5. Mitochondrial membrane structure (monitored by the probe pyrene decanoate) as a function of increasing amounts of incorporated DHA. Insert compares the excimer/monomer ratio (fluorescence intensity at 480 nm/398 nm) as a function of the amount of membrane-incorporated DHA. See Figure 1 for abbreviation.

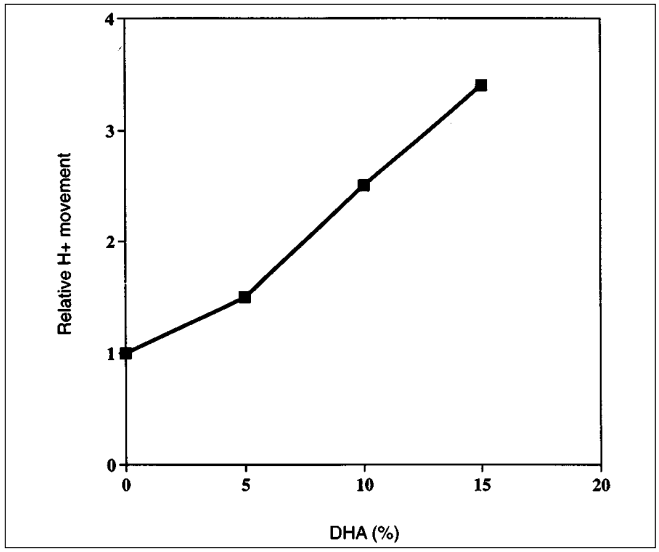

FIG. 6. Relative proton movement of small unilamellar vesicles made from mitochondrial lipid extracts as a function of incorporated 18:0/22:6 phosphatidylcholine expressed as percentage DHA. See Figure 1 for abbreviation.

into the mitochondrial membranes. The increase in E/M indicates that incorporation of DHA into the mouse mitochondrial membrane is associated with increased fluidity (Fig. 5).

Lipids that were extracted from nonfused BALB/c mitochondria were mixed with varying amounts of 18:0/22:6 PC, and SUV were made in the presence of BCECF. After removing the nonsequestered BCECF on a Sephadex G-50 column, a pH gradient was established and changes in H^+ movement monitored. In Figure 6, H^+ movement is shown to be proportional to the level of incorporated 18:0/22:6 PC while added 18:0/18:1 PC had no measurable effect (results not shown).

TABLE 1
Fatty Acid Compositions of Hydrogenated Coconut Oil and Menhaden Oil

Fatty acid	Percentage of total fatty acids: Hydrogenated coconut oil	Menhaden oil
14:0	46.2	7.9
16:0	23.1	27.0
18:0	30.1	2.7
18:1(n-9)	0	14.6
18:2(n-6)	0	2.1
18:3(n-3)	0	1.6
20:5(n-3)	0	15.9
22:6(n-3)	0	16.1

Mitochondria and dietary DHA. Mitochondria were isolated from young (5-mon-old) and old (24-mon-old) CBA/Ca mice that had been maintained on identical diets with the exception of the fat source. Half of the mice in each age group were fed HCO while the other half were given MO. Both diets contained 0.5% corn oil for essential fatty acids. HCO is rich in saturated fats while MO is rich in long-chain polyunsaturated n-3 fatty acids. The fatty acid composition of each oil is listed in Table 1.

The relative compositions of the predominant fatty acids for mitochondria isolated from the different mice are listed in Table 2. Mitochondrial fatty acid composition was markedly affected by both age and diet. As expected, after 3 wk the percentage of the n-3 long-chain fatty acids DHA and EPA (eicosapentaenoic acid) increased dramatically for mice on the MO diet while the level of arachidonic acid (20:4) decreased. To further define age and diet-related changes, we calculated the unsaturation index, that is, the sum of the amount times the number of unsaturations for each unsaturated fatty acid divided by the total amount of all fatty acids.

TABLE 2
Fatty Acid Compositions of the Three Major Phospholipids—Phosphatidylcholine, Phosphatidylethanolamine, and Cardiolipin[a]

Group	16:0	18:0	18:1	18:2	20:4	20:5	22:6
			Phosphatidylcholine				
Y-HCO	31.9	7.1	25.5	12.9	6.5	n.d.	5.9
O-HCO	26.3	9.1	25.6	9.6	11.9	n.d.	3.9
Y-MO	35.6	11.3	13.5	7.6	4.1	5.4	21.8
O-MO	26.1	13.7	17.2	8.5	4.8	7.1	20.7
			Phosphatidylethanolamine				
Y-HCO	19.6	17.3	17.4	4.6	21.4	n.d.	10.8
O-HCO	18.2	13.1	17.9	5.5	26.5	n.d.	7.1
Y-MO	14.1	24.9	13.7	14.9	4.3	3.6	24.1
O-MO	15.8	23.1	12.9	5.9	3.9	5.9	28.8
			Cardiolipin				
Y-HCO	15.4	4.5	33.9	13.1	11.8	n.d.	2.3
O-HCO	14.5	12.6	29.0	13.5	9.2	n.d.	2.0
Y-MO	17.0	11.5	17.5	13.4	3.0	4.6	16.6
O-MO	19.4	15.3	16.5	12.9	3.1	3.7	13.0

[a]Found in mitochondria taken from mice maintained on the hydrogenated coconut oil (HCO) or menhaden oil (MO) diet. Liver mitochondria were isolated from young (Y) or old (O) CBA/Ca female mice; n.d., not detected.

TABLE 3
A Comparison of Various Properties of Liver Mitochondria[a]

	Y-HCO	Y-MO	O-HCO	O-MO
ATP concentration (μg ATP/mg protein)	19.1	10.8	14.6	8.1
RCI	10.2 ± 2.65	7.93 ± 1.79	8.34 ± 1.04	5.66 ± 0.67
State 3 respiration (μM O_2/min/mg mitochondrial protein)	0.172 ± .022	0.091 ± .006	0.152 ± .017	0.137 ± .023
State 4 respiration (μM O_2/min/mg mitochondrial protein)	0.017 ± .003	0.012 ± .003	0.018 ± .004	0.024 ± .005
Swelling velocity [d(1/A)/dt%]	0.44 ± .044	0.43 ± .06	0.37 ± .072	0.31 ± .041
Molecular area ($Å^2$)	64.1 ± 3.7	75.9 ± 3.1	53.1 ± 1.2	70.4 ± 1.0

[a]Isolated from young mice maintained on the HCO diet (Y-HCO), young mice maintained on the MO diet (Y-MO), old mice maintained on the HCO diet (O-HCO), or old mice maintained on the MO diet (O-MO). A: absorbance; t: time.

The unsaturation index was much larger for the MO-fed mice than for the HCO mice [unsaturation index: young (Y)-HCO, 121; Y-MO, 265; old (O)-HCO, 165, O-MO, 187]. After 3 wk on the MO diet, both the young and old mice had high levels of DHA associated primarily with phosphatidylethanolamine (PE) and PC.

Mitochondrial bioenergetics, studied by several techniques, was shown to be affected by age. The 24-mon-old mice had much lower ATP levels, RCI, State 3 respiration rates, and phosphate-dependent swelling velocities than did the 5-mon-old mice (Table 3). For all bioenergetic properties tested, addition of MO to the diet not only failed to halt or reverse the age-dependent decrease in function, it actually amplified the decrease.

Studies with monolayers composed of mitochondrial phospholipid extracts show the phospholipid area/molecule increases with the amount of incorporated DHA for the MO diet compared to the HCO diet (Table 3). The increase in phospholipid area/molecule measured with mitochondria isolated from mice maintained on the MO diet relative to the more saturated HCO diet is consistent with the measured increase in unsaturation index. We therefore measured total lipid peroxidation in the various mitochondrial lipid extracts by a malondialdehyde assay (16). These measurements indicate that while the malondialdehyde levels do indeed increase in the MO-fed mouse mitochondria, the levels are quite low. The malondialdehyde values for mice on the various diets were: Y-HCO, 0.176 nmol/mg P; Y-MO, 0.82 nmol/mg P; O-HCO, 0.282 nmol/mg P; and O-MO, 0.407 nmol/mg P.

DISCUSSION

Although the effects of lipids on mitochondria are very complex and alterations of any fatty acid may influence function, we nevertheless have decided here to concentrate on DHA. Since DHA is the most unsaturated biological fatty acid commonly found in nature (28), it is a likely target for oxidation. One possibility is that the DHA-dependent decrease in mitochondrial function is simply the result of accumulated oxidized products (29). Indeed our prior experminents with phospholipid vesicles have demonstrated that DHA influences general membrane properties, including permeability, more than other common fatty acids (6,30). It is reasonable to propose that mitochondrial function, which is dependent on maintaining an intact barrier to H+ leakage, would be particularly susceptible to DHA-induced permeability changes, and therefore mitochondria may be an ideal model system to search for the membrane structure/function relationship of n-3 fatty acids. It was the purpose of the experiments described here to test this hypothesis by monitoring the effect of DHA on mitochondrial H^+ movement and associated bioenergetic functions.

A variety of mitochondrial functions have often been reported to be controlled, in part, by the lipid composition of the inner membrane. Lipid compositions have been experimentally manipulated by obtaining animals of different ages, putting the animals on fatty acid-deficient diets or by supplementing a minimal diet with specific fatty acids. However, there have been few unequivocal experiments linking fatty acid composition to mitochondrial function, and the literature is fraught with obvious contradictions. Chiu and Richardson (31) have suggested that reports of discrepancies are the consequence of comparing mitochondria isolated from different organisms or different tissues from the same organism, or organisms of different age, or use of different substrates employed in the bioenergetic measurements. A survey of the literature suggests that with increasing age there is a decrease in the number of mitochondria per cell (32,33). Also age-related decreases in the activity of many mitochondrial enzymes (33–35), most notably those involved in translocation, have been reported (23,36–38). Decreases in respiratory and bioenergetic activities (37,39–42) and increases in superoxide radicals (41,43) have been described in older mitochondria. Finally structural and compositional membrane changes have been demonstrated as mitochondria age. Old mitochondria have enhanced permeability (44,45) that is likely related

to cholesterol (37,46) and cardiolipin (46) content, saturated/unsaturated fatty acid ratios (35,41,47), cholesterol/phospholipid ratios (37,46,48), PC/PE ratios (37), and n-6/n-3 fatty acid ratios (37). The reported changes in lipid content associated with aging may be partially responsible for concomitant changes in membrane potential (44) and fluidity (33–35,46,47).

Among the many membrane-associated processes known to be affected by age (49), bioenergetic events occurring at the mitochondrial inner membrane are of particular interest. Alterations in membrane lipid composition of older mitochondria may be related to their gross structural abnormalities (50,51), and changes in membrane potential (44), organization, fluidity (52), and permeability (45). The aged cell's reduced ability to produce ATP may be related to these changes (37,39–42). Because of the age-related reduction in mitochondrial function, Harman (33) has proposed that mitochondria may act as "biological clocks."

Many reports have indicated that mitochondrial lipid composition is susceptible to dietary manipulation. It has been shown that mitochondrial fatty acid profiles resulting from fatty acid deficiency can be rapidly restored through diet (53). Tahin *et al.* (53) reported maximal uptake of a variety of dietary fatty acids into mitochondrial membranes in less than 3 wk, while Yamaoka *et al.* (54) reported maximal fatty acid changes in PC and PE after only 10 d of dietary sardine oil. Most of the same properties reported to change upon aging have also been shown to be altered through diet (55,56). However, as with the aging studies, there is considerable controversy as to whether dietary fatty acids actually alter mitochondrial bioenergetics. Reports demonstrating dietary fatty acid-induced decreases in RCI, State 3 and State 4 respiration and P/O ratios (54,57–59) are offset by reports demonstrating no or contradictory effects on the same properties under similar conditions (60–62).

Since it is generally (although not universally) accepted that aging results in significant decreases in many mitochondrial activities that are related to changes in the lipid component of the mitochondrial inner membrane, and mitochondrial lipid composition can be readily altered through diet, Barzanti *et al.* (63) suggested that perhaps dietary lipids can significantly modify the age-related decline in mitochondrial activity.

The experiments reported here confirm that mitochondrial bioenergetic activity does in fact decrease with age (Table 3). We also confirm that mitochondrial fatty acid profiles can be rapidly altered by dietary fat and that n-3 fatty acids, in particular DHA, can be effectively accumulated into mitochondria of young and old mice (Table 2). While incorporation of DHA does alter lipid bilayer physical properties as detected by the fluorescence probes DPH (results not shown), MC540 (Fig. 4), pyrene decanoate (Fig. 5) and area/molecule measurements, it did not enhance any of the bioenergetic properties tested. In fact, all bioenergetic properties tested appeared to decrease upon DHA incorporation.

Ex vivo experiments enhancing DHA levels through diet were complemented *in vitro* by experiments in which 18:0/22:6 PC SUV were mixed with mitochondria. Upon incorporation of 18:0/22:6 PC into mouse mitochondria, a marked decrease in RCI associated with an increase in H^+ movement and decrease in membrane potential were measured (Fig. 3). The DHA-induced increase in H^+ movement may be due to changes in the lipid bilayer component of the mitochondrial membrane, since SUV made from mitochondrial lipid extracts also demonstrate a parallel DHA-induced enhancement in H^+ movement (Fig. 6). The fluorescent membrane probes DPH, MC540, and pyrene also support this conclusion by indicating changes in mitochondrial membrane bilayer structure induced by DHA (Figs. 4 and 5).

Complete explanations for changes in complex biological processes such as oxidative phosphorylation are difficult. We have explained the DHA-dependent decreases in bioenergetic activities to be, in part, the result of membrane perturbations caused by DHA. This seems reasonable in light of the work of Brand and colleagues (64,65) on the effect of lipid composition on mitochondrial H^+ leakage. We realize, however, that other interpretations including extensive lipid peroxidation, increases in phospholipase A_2 activity (66), changes in adenylate cyclase activity (67), and alterations in prostanoid levels may also play a role.

We conclude that although mitochondrial membrane composition can be easily altered with dietary n-3 fatty acids, changing mitochondrial membranes in this way does not alter bioenergetic function in a manner likely to reverse the deleterious effects of age.

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Molecular Species of Phosphoglycerides in Liver Microsomes of Rats Fed a Fat-Free Diet

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ABSTRACT: The influence of a fat-free diet on the molecular species composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) of rat liver microsomes was studied by using reversed-phase high-pressure liquid chromatography. In the three phosphoglyceride classes analyzed, the fat-free diet produced a large decrease in the 18:0/20:4n-6 species but less important changes were found in the 16:0/20:4n-6 species. In PC, the most abundant phosphoglyceride class of rat liver microsomes, the fall in the 18:0/20:4n-6 species was counterbalanced mainly by an enhancement in the 16:0/18:1n-9 species although it was not evident in PE. In PI, the decrease in the 18:0/20:4n-6 species was counterbalanced by an increase in the 18:0/20:3n-9 species. Fluorescence polarization measurements of 1,6-diphenyl-1,3,5-hexatriene in liposomes of 16:0/18:1n-9-, 18:0/18:1n-9-, 16:0/20:4n-6-, and 18:0/20:4n-6-PC indicated that the change in the saturated fatty acid in the *sn*-1 position accompanying the replacement of 20:4n-6 by 18:1n-9 could be very important for a homeoviscous compensation, maintaining the membrane physical properties without large alterations in spite of the essential fatty acid deficiency due to the fat-free diet.
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The major function of essential fatty acids (EFA) is to be precursors of biooxygenated derivatives such as eicosanoids and docosanoids. However, only a small fraction of the requirements of these acids in the mammal diet can be explained by this role (1). Since large amounts of these acids and their derivatives are present in membrane phospholipids, a structurally specific role in the membranes has been also attributed to these acids (2,3). Although some authors have minimized the importance of EFA for the structural properties of membranes (4), others have suggested that they could modify the conformational freedom of the acyl chains of lipid bilayers with participation in lipid–protein interactions and membrane function (5,6). The influence of unsaturation on the bilayer structural and dynamic properties is strongly dependent on the position of double bonds (7–9). Since only EFA and their derivatives are able to place double bonds in the deep part of the membrane leaflet (3,10,11), their deficiency could result in altered transversal double-bond distribution and physical properties of the membranes.

EFA-deficient and fat-free diets modify the fatty acid composition of several membranes in different species (3,10–17). Although it depends to some extent on the particular membrane, tissue and species, these diets produce, in general, a fall in the n-6 fatty acids 18:2 and 20:4 and an increase in the nonessential 18:1n-9 fatty acid. Appreciable amounts of the nonessential fatty acid 20:3n-9, which is present only in trace amounts in EFA-sufficient animals, appear as a consequence of EFA or fat-free diets in rat (10,12,13) but not in guinea pig liver microsomes (14–17).

Several studies using spectroscopic methods such as fluorescence (14,16,17) or electron spin resonance (3,6,15) have indicated that EFA or fat-free diets affect the physical properties of different membranes, such as guinea pig liver microsomes (14–17) or piglet jejunum brush border membranes (3,6). This is attributed to changes in the fatty acid composition of membrane lipids, since no changes are observed either in the phospholipid class distribution or in the relative content of cholesterol, phospholipid, and protein. In some membranes, however, as is the case for rat erythrocyte (10) and liver microsomal (10,13) membranes, in spite of large changes in fatty acid composition, membrane lipid order and dynamics remain unaltered, suggesting that some homeoviscous compensation could occur (10). As already shown in artificial membranes of synthetic phosphatidylcholines (PC), the influence of an unsaturated fatty acid in the *sn*-2 position on the viscotropic properties is largely dependent on the saturated one in *sn*-1 (9). Thus, to understand the mechanism of this homeoviscous compensation, it was necessary to study the changes in the phospholipid molecular species resulting from a fat-free diet. This should supply more detailed information than the fatty acid compositional changes.

This study shows that the largest change evoked by a fat-free diet in rat liver microsomes is the replacement of

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Abbreviations: DPH, 1,6-Diphenyl-1,3,5-hexatriene; EFA, essential fatty acids; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; UV, ultraviolet.

18:0/20:4n-6-PC by 16:0/18:1n-9-PC. Fluorescence anisotropy measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH) in liposomes of these PC in comparison with 16:0/20:4n-6- and 18:0/18:1n-9-PC suggest that the replacement in the *sn*-1 position of 18:0 by 16:0 could be very important for the homeoviscous compensation observed in liver microsomes of rats fed on the fat-free diet.

EXPERIMENTAL PROCEDURES

Materials. DPH was obtained from Aldrich Chemical Co. (Milwaukee, WI). A stock solution of 2.0 mM in tetrahydrofuran was prepared. The 16:0/18:1n-9-, 18:0/18:1n-9-, 16:0/20:4n-6-, and 18:0/20:4n-6-PC (dissolved in chloroform) were obtained from Avanti Polar Lipids (Alabaster, AL).

Animals and diets. After weaning, male Wistar rats were fed either a control diet: 52% starch, 19% casein, 23% sunflower oil plus minerals (4%) and vitamins (2%) (18) or a fat-free diet: 66% starch, 28% casein, minerals (4%) and vitamins (2%). The rats were killed by decapitation either one or three months later, and the livers were excised.

Microsome preparation. Livers were homogenized in 0.25 M sucrose, 1 mM EDTA, pH 7.0. Microsomes were obtained by centrifugation as previously described (19) and stored at −80°C. The protein content of microsomal preparations was measured by the method of Lowry *et al*. (20).

Extraction and separation of lipid fractions. Total microsomal lipids were extracted according to the procedure of Folch *et al*. (21) with chloroform/methanol 2:1 (vol/vol). Glycerophospholipid classes were separated by one-dimensional thin-layer chromatography on 20×20 cm plates coated with a mixture of silica gel H–florisil 9:1 (w/w) with a double development solvent system (22): chloroform/methanol/30% ammonium hydroxide/water 140:50:7:3 (by vol) in the first run and chloroform/methanol/acetic acid/water 320:40:8:3 (by vol) in the second run. The extreme ends of the plates were sprayed with an iodine solution to visualize the spots. The unexposed part of the bands corresponding to PC, phosphatidylethanolamine (PE), and phosphatidylinositol (PI) were scraped off and extracted with methanol/chloroform 2:1 (vol/vol).

Fatty acid composition analysis. After evaporation of the solvent under vacuum, the total lipid extracts and the different phospholipid fractions were transesterified by heating at 64° for 3 h in 10% boron trifluoride in methanol (from Sigma Chemical Co., St. Louis, MO). The resulting methyl esters were separated on a column of 10% SP 2330 on 100–120 mesh Chromosorb WAW (Supelco, Bellefonte, PA) using a Hewlett-Packard 5840 A gas–liquid chromatograph (Palo Alto, CA). The fatty acids were identified by comparison of their relative retention times with those of reference standards.

Phospholipid molecular species analysis. The analysis of phospholipid molecular species was essentially made as described by Patton *et al*. (23) with some modifications. PC, PE, and PI molecular species were separated on a 4×250 mm Bio-Sil ODS-5S column (Bio-Rad, Richmond, CA), eluted with 50 mM choline chloride in methanol/water/acetonitrile 90:7.5:8 (vol/vol) at a flow rate of 1.5 mL/min using a Merck-Hitachi L-6200 pump (Darmstadt, Germany). Detection was performed by absorption at 205 nm using a Merck-Hitachi L-4200 ultraviolet–visible (UV-VIS) detector. For the identification and quantification of the molecular species, 400 µg of each phosphoglyceride were injected onto the column. Individual peaks were collected and mixed with 40 µg of di-20:1(n-9)-PC used as internal standard. They were extracted with chloroform and after the evaporation of the solvent under vacuum; they were transesterified as described above for the fatty acid analysis. Gas–liquid chromatography (GLC) analysis of each high-performance liquid chromatography (HPLC) peak made possible the identification of the fatty acids which were present in each molecular species. The relative GLC–flame-ionization detection (FID) response with respect to the internal standard 20:1 made possible the quantification of each molecular species. Relative response factors for the HPLC UV absorption detection were calculated for each molecular species of phosphoglyceride. These factors were used to calculate the composition in molecular species of the individual samples directly from the HPLC chromatograms obtained by injecting 200-µg phosphoglyceride samples.

Fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene. Multilamellar liposomes of 16:0/18:1-, 18:0/18:1-, 16:0/20:4, and 18:0/20:4-PC were prepared in 20 mM sodium phosphate pH 7.1 using the procedure described by Kutchai *et al*. (24). For labeling with DPH, 2 mL of a 2.5 µM probe dispersion in 20 mM phosphate buffer pH 7.1 were mixed with the same volume of liposome preparations, agitated, and incubated at 40°C for 30 min. All procedures were carried out under N_2 atmosphere. For background corrections, unlabeled blanks with the same concentration of liposomes were also prepared (25).

Steady-state anisotropy was measured at 20°C in an SLM 4800 C spectrofluorometer (SLM Instruments, Inc., Urbana, IL) as described (26). The excitation wavelength was 361 nm, and the emission was observed through a sharp cut-off filter (Schott KV 389; Glass Technologies, Inc., Duryea, PA).

RESULTS

Table 1 shows the effect of a 1-mon treatment with a fat-free diet on the fatty acid composition of rat liver microsomes. This is in agreement with our previous findings (10,13). The fat-free diet resulted in a highly significant decrease in the n-6 acids 18:2 and 20:4. With respect to the minor n-3 acids, although a significant redistribution was produced by the treatment, the total amount was not changed. The decrease in n-6 acids was counterbalanced by a rise in the nonessential n-9 monoenoics 16:1 and 18:1 and the trienoic 20:3n-9. An increase in 16:0 was compensated by a decline in 18:0, and no net change in the total saturated acid content occurred. In this way, the ratio of unsaturated to saturated acyl chains was not

TABLE 1
Effect of a Fat-Free Diet on the Fatty Acid Composition of the Total Lipids and the Major Phosphoglyceride Classes of Rat Liver Microsomes

	Total lipids		Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylinositol	
Fatty acid	Control diet (% area)[a]	Fat-free diet (% area)	Control diet (% area)	Fat-free diet (% area)	Control diet (% area)	Fat-free diet (% area)	Control diet (% area)	Fat-free diet (% area)
16:0	14.7 ± 0.4	21.7 ± 2.4**	19.6 ± 3.0	23.9 ± 3.5*	13.4 ± 1.2	20.0 ± 3.4**	9.0 ± 2.6	10.7 ± 2.6[ns]
16:1n-7	0.5 ± 0.3	6.9 ± 0.9***	0.4 ± 0.4	6.3 ± 1.3***	n.d.[c]	2.5 ± 0.8**	0.5 ± 1.2	0.6 ± 0.6[ns]
18:0	18.6 ± 0.3	13.1 ± 2.1**	18.6 ± 2.3	11.8 ± 2.3**	21.4 ± 1.8	14.9 ± 1.5***	37.6 ± 2.6	37.8 ± 4.9[ns]
18:1n-9	12.6 ± 0.7	22.6 ± 1.8***	11.4 ± 1.0	22.5 ± 2.4***	10.6 ± 1.4	17.4 ± 2.2***	6.6 ± 2.0	8.1 ± 3.8[ns]
18:2n-6	16.5 ± 0.8	4.7 ± 0.5***	14.5 ± 1.6	6.1 ± 0.6***	10.3 ± 1.2	3.3 ± 0.5***	5.4 ± 1.5	1.6 ± 0.6**
20:3n-9	1.1 ± 0.2	4.1 ± 0.8**	1.8 ± 0.5	3.8 ± 1.4*	1.7 ± 0.6	2.5 ± 0.8ns	1.3 ± 0.9	11.7 ± 1.7***
20:3n-6	0.9 ± 0.1	2.7 ± 0.4***	0.7 ± 0.3	2.8 ± 0.7**	0.7 ± 0.4	1.5 ± 0.5*	0.5 ± 0.2	1.9 ± 1.2*
20:4n-6	25.1 ± 0.8	14.9 ± 1.1***	23.9 ± 3.9	13.7 ± 1.4**	24.6 ± 0.7	21.5 ± 0.8***	34.7 ± 1.7	21.5 ± 3.7***
22:4n-6	2.8 ± 0.5	1.4 ± 0.4*	1.8 ± 0.6	0.8 ± 0.5*	4.0 ± 0.9	1.2 ± 0.6***	1.1 ± 0.7	0.9 ± 0.3[ns]
22:4n-3[b]	4.6 ± 0.8	3.9 ± 0.8[ns]	4.5 ± 0.9	4.1 ± 0.9[ns]	8.3 ± 1.4	6.6 ± 1.7[ns]	2.1 ± 1.2	3.2 ± 1.5[ns]
22:5n-3	0.6 ± 0.2	0.9 ± 0.4[ns]	0.7 ± 0.4	0.7 ± 0.3[ns]	1.2 ± 0.6	1.8 ± 1.7[ns]	0.5 ± 0.7	0.4 ± 0.3[ns]
22:6n-3	1.8 ± 0.4	3.0 ± 0.4**	2.1 ± 1.3	3.3 ± 1.2[ns]	3.6 ± 1.1	6.7 ± 2.2*	0.7 ± 0.6	1.5 ± 0.4*
U/S[d]	1.8 ± 0.2	1.7 ± 0.4[ns]	1.5 ± 0.4	1.6 ± 0.4[ns]	1.7 ± 0.3	1.6 ± 0.4[ns]	1.1 ± 0.3	1.0 ± 0.4[ns]

[a]Data are the mean of five animals for each group ± standard deviation. [b]Contains also 22:5n-6. [c]Nondetectable amount. [d]Molar ratio of unsaturated to saturated fatty acids; [ns]not significant difference; *$P < 0.1$; **$P < 0.01$; *** $P < 0.001$.

significantly altered. After 3 mon of the fat-free diet treatment, the same changes were basically found in the fatty acid composition (not shown).

Table 1 also shows the effect of a 1-mon treatment with a fat-free diet on the fatty acid composition of the three major phosphoglyceride classes of rat liver microsomes: PC, PE, and PI. Although microsomes contain minor phospholipid classes such as phosphatidylserine and sphingomyelin, PC, PE and PI make more than 80% of the microsomal lipids. The changes in the fatty acid composition produced by the fat-free diet were different in each one of these phospholipid classes. A decrease in 18:2n-6 was produced in all the phospholipid classes, but there was no large decrease in 20:4n-6 in the PE fraction as a result of fat deprivation. The highest increase in monoenoic acids was found in the PC fraction. In the PI fraction containing the largest amount of 20:4n-6 but the smallest amount of 18:2n-6, the fat-free diet produced the highest increase in the nonessential trienoic 20:3n-9 and no increase in monoenoic acids. As in the total microsomal lipids, the 3-mon treatment resulted in similar changes in the fatty acid composition of each phospholipid class (not shown) as those observed for the 1 mon-treatment.

Figure 1 shows typical chromatograms obtained for the HPLC separation of the molecular species of PC, PE, and PI from liver microsomes of rats fed on a control or a fat-free diet. In the PC and PE fractions of fat-fed animals, eight major molecular species were observed. Two new species containing 20:3n-9 became detectable in animals fed the fat-free diet. Only two major molecular species (16:0/20:4n-6 and 18:0/20:4n-6) were observed in the PI fraction of control animals, and 18:0/20:3n-9 became observable in the animals fed the fat-free diet.

Table 2 shows changes produced by the fat-free diet on the composition in molecular species of the major phosphoglyceride classes from rat liver microsomes. The two major molecular species in PC, PE, and PI were those containing arachidonic acid (16:0/20:4n-6 and 18:0/20:4n-6). The fat-free diet produced a marked decrease in the 18:0/20:4n-6 species of the three phosphoglycerides analyzed. However, in the case of 16:0/20:4n-6, the fat-free diet evoked only a small decrease in the PC fraction, a small increase in the PE fraction and no significant change in the PI fraction. A second major change produced by the fat-free diet was a large increase in 16:0/18:1n-9-PC. The amount of 16:0/18:1n-9 species of PE, however, was not significantly changed by the treatment. The nonessential polyenoic 20:3n-9 fatty acid, which appeared as a consequence of the fat deficiency, was found in two molecular species of PC and PE (16:0/20:3n-9 and 18:0/20:3n-9) but in only one species of PI (18:0/20:3n-9). Although the fat-free diet produced a significant decrease in the content of the 18:2n-6 fatty acid in PC and PE (see Table 1), no significant changes were observed in the 16:0/18:2n-6 and 18:0/18:2n-6 species of these phosphoglycerides. This fact could be due to the presence of other minor molecular species which were not detected by the HPLC analysis.

In accordance with previous studies (19), the fat-free diet did not evoke significant changes in the phospholipid class distribution and in the lipid/protein or cholesterol/phospholipid ratios of rat liver microsomes (not shown). The PC, PE, and PI contents of both groups of liver microsomes were 0.32, 0.15, and 0.055 μmol/mg of protein, respectively. The amount of each phosphoglyceride molecular species (in nmol/mg of protein) was calculated and is given in Table 2. It shows that the major change produced by the fat-free diet was the decrease in 18:0/20:4n-6 PC, which was mainly replaced by 16:0/18:1n-9.

It was our interest to know if the change in the saturated fatty acid in *sn*-1 (18:0 by 16:0) which accompanies the change in the unsaturated one in *sn*-2 (20:4n-6 by 18:1n-9) could play some role in the compensation observed in the fat-deficient condition. For this reason, steady-state fluorescence anisotropy of DPH in multilamellar liposomes of 18:0/20:4

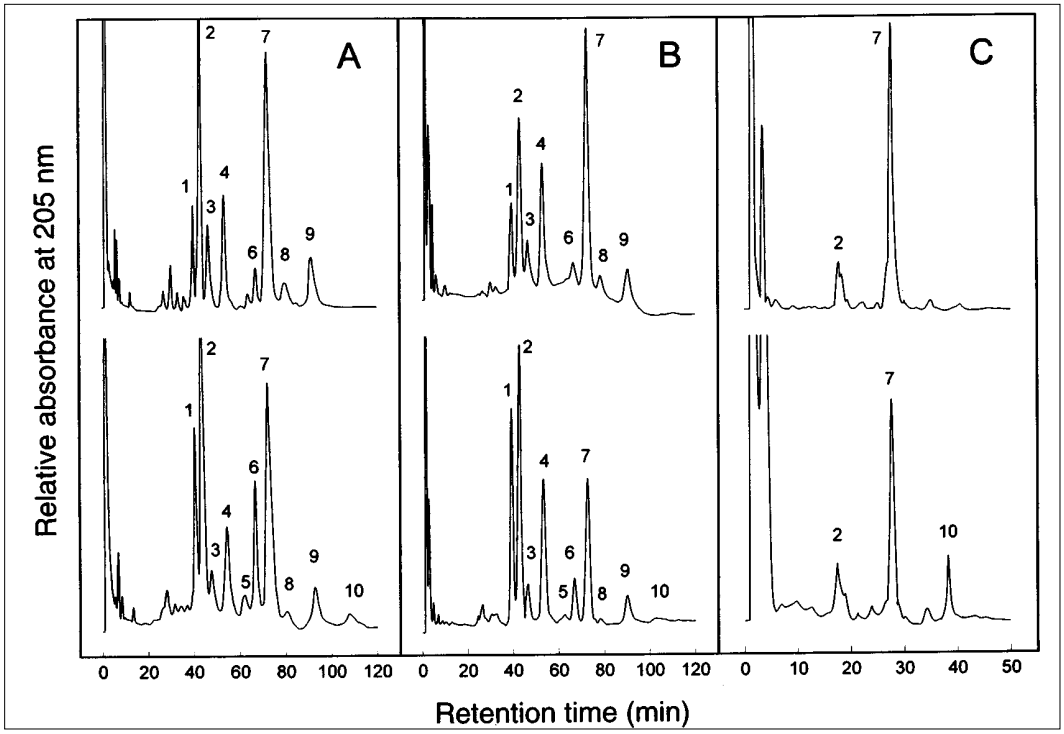

FIG. 1. Reversed-phase high-performance liquid chromatographic separation of molecular species of phosphatidylcholine (A), phosphatidylethanolamine (B), and phosphatidylinositol (C) of liver microsomes from rats fed on a control (top chromatograms) or a fat-free (bottom chromatograms) diet. About 200 µg of the corresponding lipids were chromatographed as described in the Experimental Procedures section. Identified peaks are numbered in sequence of elution: 1, 16:0/22:6n-3; 2, 16:0/20:4n-6; 3, 16:0/18:2n-6; 4, 16:0/22:5n-3; 5, 16:0/20:3n-9; 6, 16:0/18:1n-9; 7, 18:0/20:4n-6; 8, 18:0/18:2n-6; 9, 18:0/22:5n-3; and 10, 18:0/20:3n-9.

n-6-, 18:0/18:1n-9-, 16:0/20:4n-6- and 16:0/18:1n-9 PC, was measured. Figure 2 shows that fluorescence anisotropy was lower in liposomes of the 20:4n-6-containing species compared with those of the 18:1n-9-containing species. Fluorescence anisotropy was higher in liposomes of the 18:0-containing PC than in those of the 16:0-containing ones. The increase in fluorescence anisotropy produced by the change of 20:4n-6 for 18:1n-9 in *sn*-2 was largely compensated by the change of 18:0 for 16:0 in the *sn*-1 position.

DISCUSSION

Differential polarized phase fluorescence studies dealing with probes of different localization in the lipid bilayer, using either two fixed frequencies (10) or multiple modulation frequencies for the excitation (27), indicated that the order and dynamics of the lipid bilayer of rat liver microsomes are not significantly modified by the EFA deficiency produced by fat-free diets. Only a small decrease in the rate of dipolar relaxation at the interfacial level, where phospholipid polar groups are located, was detected using Laurdan as probe (27). These studies suggest that changes in the fatty acid composition produced by fat deficiency would occur in such a way that the lipid bilayer properties are largely compensated.

The present work shows that a fat-free diet affects differentially the composition in molecular species of the main phosphoglyceride classes of rat liver microsomes and that the changes in the unsaturated fatty acids of *sn*-2 are accompanied by changes in the saturated acids in *sn*-1. In the three phosphoglyceride classes analyzed, fat deficiency produced a large decrease in the 18:0/20:4n-6 species but small changes in the 16:0/20:4n-6 species. In PC, the most abundant phosphoglyceride class of rat liver microsomes, the decrease in the 18:0/20:4n-6 species was mainly counterbalanced by an increase in the 16:0/18:1n-9 species. However, in PI, the decrease in the 18:0/20:4n-6 species was counterbalanced by an increase in the 18:0/20:3n-9 species.

Since the fat-free diet evoked no change in the phospholipid class distribution and PC is the major lipid class of rat liver microsomes, the replacement of 18:0/20:4n-6 PC by 16:0/18:1n-9 PC was the major change produced by the treatment. These results also show that the increase in the fluorescence steady-state anisotropy of DPH due to the change of 20:4n-6 by 18:1n-9 in the *sn*-2 position of PC was largely compensated by the change of 18:0 by 16:0 in the *sn*-1 position. This fact indicates that the change in the saturated fatty acid in *sn*-1 accompanying the replacement of 20:4n-6 by 18:1n-9 in *sn*-2 could be very important for a homeoviscous compensation, maintaining the membrane physical proper-

TABLE 2
Effect of a Fat-Free Diet on the Composition in Molecular Species of the Major Phosphoglyceride Classes of Rat Liver Microsomes

	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylinositol	
Molecular species	Control diet mol%[a] (nmol/mg)[b]	Fat-free diet mol% (nmol/mg)	Control diet mol% (nmol/mg)	Fat-free diet mol% (nmol/mg)	Control diet mol% (nmol/mg)	Fat-free diet mol% (nmol/mg)
16:0/22:6n-3	2.3 ± 0.3 (7.4 ± 1.0)	5.9 ± 1.4** (18.9 ± 4.5)	4.5 ± 0.2 (7.2 ± 0.3)	9.3 ± 1.7** (14.9 ± 2.7)	n.d.	n.d.
16:0/20:4n-6	25.9 ± 2.2 (82.9 ± 7.0)	19.7 ± 3.8* (63.0 ± 12.2)	17.0 ± 2.4 (27.2 ± 3.8)	22.0 ± 3.7* (35.2 ± 5.9)	18.4 ± 8.7 (10.1 ± 4.8)	17.2 ± 6.9[ns] (9.5 ± 3.8)
16:0/18:2n-6	17.8 ± 3.3 (57.0 ± 10.6)	15.0 ± 2.3[ns] (48.0 ± 7.4)	12.6 ± 1.5 (20.2 ± 2.4)	13.8 ± 4.1[ns] (22.1 ± 6.6)	n.d.	n.d.
16:0/22:5n-3	6.2 ± 0.6 (19.8 ± 1.9)	8.1 ± 0.9** (25.9 ± 2.9)	11.7 ± 1.0 (18.7 ± 1.6)	10.2 ± 2.1[ns] (16.3 ± 3.4)	n.d.	n.d.
16:0/20:3n-9	n.d.[c]	5.6 ± 1.5** (17.9 ± 4.8)	n.d.	1.8 ± 2.6[ns] (2.9 ± 4.2)	n.d.	n.d.
16:0/18:1n-9	11.6 ± 3.2 (37.1 ± 10.2)	22.5 ± 1.9*** (72.0 ± 6.1)	15.3 ± 5.0 (24.5 ± 8.0)	18.1 ± 2.7[ns] (29.0 ± 4.3)	n.d.	n.d.
18:0/20:4n-6	27.0 ± 3.3 (86.4 ± 10.6)	10.9 ± 2.8*** (34.9 ± 9.0)	30.1 ± 1.8 (48.2 ± 2.9)	15.0 ± 2.9*** (24.0 ± 4.6)	81.6 ± 8.7 (44.9 ± 4.8)	54.3 ± 5.8*** (29.9 ± 3.2)
18:0/18:2n-6	6.7 ± 1.4 (21.4 ± 4.5)	6.5 ± 3.0[ns] (20.8 ± 9.6)	2.9 ± 0.7 (4.6 ± 1.1)	2.2 ± 2.2[ns] (3.5 ± 3.5)	n.d.	n.d.
18:0/22:5n-3	2.6 ± 0.6 (8.3 ± 1.9)	3.6 ± 1.5[ns] (11.5 ± 4.8)	5.8 ± 1.0 (9.3 ± 1.6)	3.7 ± 0.2** (5.9 ± 0.3)	n.d.	n.d.
18:0/20:3n-9	n.d.	2.2 ± 1.0** (7.0 ± 3.2)	n.d.	3.8 ± 3.7* (6.1 ± 5.9)	n.d.	28.5 ± 8.3** (15.7 ± 4.6)

[a]Data are the mean of five animals for each group ± standard deviation. [b]Absolute amount in nmol/mg of microsomal protein. [c]Nondetectable amount.
[ns]Not significant difference; *$P < 0.1$; **$P < 0.01$; ***$P < 0.001$.

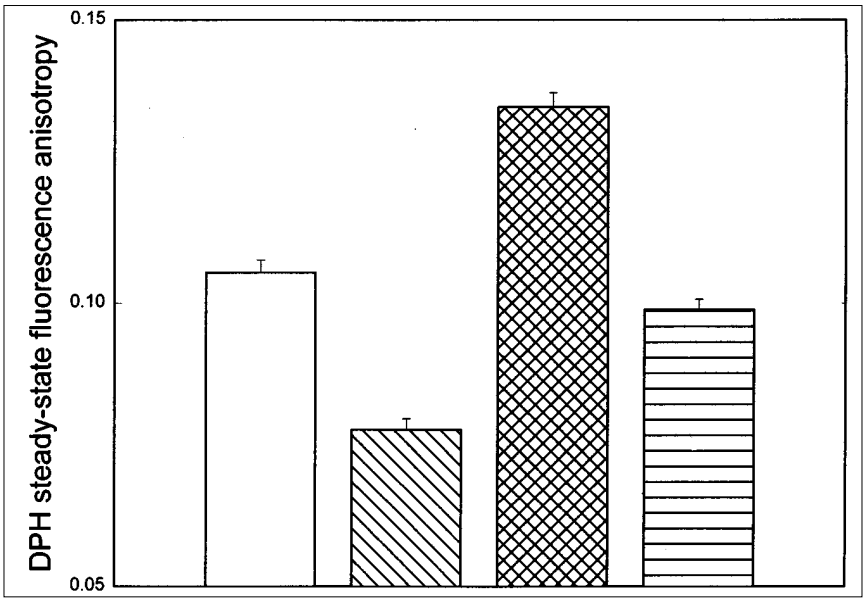

FIG. 2. Steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) in multilamellar liposomes of 16:0/18:1n-9- (open bar), 16:0/20:4n-6- (diagonally-lined bar), 18:0/18:1n-9- (cross-hatched bar), and 18:0/20:4n-6-phosphatidylcholine (vertically-lined bar).

ties without large alterations under the fat-deficient condition.

Differential polarized phase fluorescence studies using several probes of different localization in the lipid bilayer were previously made in multilamellar liposomes of these PC (9). They showed that order and viscous resistance to motion in the external bilayer region sensed by 2- and 7-(9-anthroyloxy) stearic acids are similar for 16:0/18:1 PC and 18:0/20:4 PC liposomes. However, in 18:0/20:4 PC liposomes, the deep region of the bilayer is somewhat more disordered, allowing a faster motion to 12-(9-anthroyloxy) stearic acid than in the case of 16:0/18:1 PC liposomes (9). This indicates that, although a large compensation in the microsomal membrane viscotropic properties is produced by the replacement of 18:0/20:4n-6 PC for 16:0/18:1n-9 PC, it is not enough to reach the almost exact compensation observed in fat deficiency (10). Table 2 shows that relatively minor but significant changes in other phosphoglyceride molecular species were produced by the fat-free diet in addition to the pronounced changes in 18:0/20:4n-6 PC and 16:0/18:1n-9 PC. Several of these relatively minor changes could help maintain unaltered the dynamic properties of rat liver microsomes in fat deficiency. The influence of the double-bond number and position in *sn*-2 on the acyl chain dynamics at different bilayer depths seems to be complex and seems to depend on the fatty acid in *sn*-1. In order to learn the role of these relatively minor changes for the viscotropic compensation in the fat-free condition, further measurements in liposomes of these individual phosphoglyceride species are necessary.

Another important subject is the metabolic origin of the phosphoglyceride molecular species. The 18:0/20:4n-6 PC is known to originate appreciably by methylation of PE (28), whereas the 16:0/18:1n-6 PC mainly comes from the *de novo* path. Since PE methylation seems to be sensitive to the membrane lipid dynamics (28), an interesting question to resolve in the future is whether the fat-free diet might alter the flux along the different paths.

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The Effect of Dietary Lipid on Polyunsaturated Fatty Acid Metabolism in Atlantic Salmon *(Salmo salar)* Undergoing Parr-Smolt Transformation

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ABSTRACT: The aim of this study was to measure the changes in lipid metabolism which occur during smoltification and seawater transfer in Atlantic salmon *(Salmo salar)*. Duplicate groups of Atlantic salmon parr were fed diets containing either fish oil (FO) or a blend of linseed and rapeseed oils, vegetable oil (VO), from October (week 0) to seawater transfer in May (week 26). From May to August (weeks 26–43), all fish were fed a fish oil-containing diet. Fatty acyl desaturation and elongation activity were followed in isolated hepatocytes incubated with radioactive 18:3n-3 and 18:2n-6. Metabolism of 18:3n-3 was consistently around 5-fold greater than metabolism of 18:2n-6, and total metabolism of both substrate polyunsaturated fatty acids (PUFA) was increased in fish fed both VO and FO up to seawater transfer after which desaturation activities were reduced. Desaturation activities with both 18:3n-3 and 18:2n-6 were significantly greater in fish fed VO, compared to fish fed FO, at 22 and 26 wk. Arachidonic acid (20:4n-6; AA) in liver polar lipids (PL) of fish fed VO increased consistently from weeks 0–22 but varied after seawater transfer. In fish fed FO, AA in liver PL remained constant up to week 17 before increasing at seawater transfer and leveling off thereafter. Eicosapentaenoic acid (20:5n-3; EPA) in liver PL of fish fed VO decreased significantly from week 0–22 before rising at seawater transfer and increasing rapidly posttransfer. EPA in liver PL of fish fed FO showed a similar trend except EPA was always greater in the freshwater phase compared to fish fed VO. Docosahexaenoic acid (DHA) levels in liver PL of fish fed VO remained constant in the freshwater phase before increasing following seawater transfer. In fish fed FO, DHA in liver PL increased from weeks 0–17 reducing and leveling off postseawater transfer. The levels of $PGF_{2\alpha}$ and $PGF_{3\alpha}$ were measured in isolated gill cells stimulated with calcium ionophore A23187. $PGF_{2\alpha}$ production in fish fed VO increased significantly between 0–7 wk before decreasing toward seawater transfer. After transfer, $PGF_{2\alpha}$ production increased to a peak at 35 wk. $PGF_{2\alpha}$ production in fish fed FO was not significantly altered during the trial period. The changes in $PGF_{3\alpha}$ production were broadly similar to those occurring with $PGF_{2\alpha}$, but the latter was always in excess of the former (2- to 4-fold). Plasma chloride concentrations in fish subjected to seawater challenge at 20 wk were significantly lower in fish fed VO compared to those fed FO. This study has provided new information on the changes in lipid metabolism which accompany parr-smolt transformation and suggests that diets which have a fatty acid composition more similar to that in aquatic invertebrates may be beneficial in effecting successful seawater adaptation.

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The fatty acid compositions of fish tissues are determined both by the type of dietary lipid ingested and the ability of the individual fish species to modify that dietary input *via* both catabolism and by pathways of desaturation and elongation (1,2). Freshwater fish appear able to elongate and desaturate 18:3n-3 to 22:6n-3 (3) whereas marine fish, which lack or have a very low activity of Δ5-desaturase, require the long-chain polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (20:5n-3; EPA), and docosahexaenoic acid (22:6n-3; DHA) to be supplied by the diet (4). The low Δ5 desaturase activity in marine fish reflects the abundance of EPA and DHA in the marine food chain (5), which makes a Δ5-desaturase activity largely redundant, while the freshwater food chain, which tends to lack DHA but is rich in the C_{18} PUFA 18:2n-6 and 18:3n-3, requires fish to desaturate and elongate C_{18} precursor fatty acids to form physiologically important PUFA, such as DHA (6,7).

The Atlantic salmon (*Salmo salar)* is an anadromous fish which begins life in freshwater and migrates to the sea after 1–2 yr, before returning to freshwater to breed after spending 1–3 yr at sea. The seaward migration of salmonid juveniles, or parr, is accompanied by profound changes in morphology, behavior and physiology, known collectively as parr–smolt transformation or smoltification (8). The process involves activation of neuroendocrine and endocrine systems which re-

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Abbreviations: AA, arachidonic acid; BHT, butylated hydroxytoluene; DHA, docosahexaenoic acid; EFA, essential fatty acid; EPA, eicosapentaenoic acid; FAF-BSA, fatty acid free bovine serum albumin; FO, fish oil; HBSS, Hank's balanced salt solution; MHM, Marine Harvest McConnell Ltd.; PGF, prostaglandin F; PL, polar lipid; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography; VO, vegetable oil.

sults in biochemical reorganizations within the tissues which prepare the fish, while still in freshwater, for life in the marine environment (9,10). Studies with the steelhead trout (*Oncorhynchus mykiss)* and the masu salmon (*O. masou)* suggest that the change in tissue fatty acid compositions from a typical freshwater pattern, which is relatively low in PUFA, to a typical marine pattern which is relatively rich in long-chain PUFA particularly EPA and DHA, occurs as a preadaptive response to seawater entry (11,12). In addition, Li and Yamada (12) found that masu salmon which had undergone smoltification, but which were maintained in freshwater on the same diets, underwent a desmoltification process which was accompanied by a reversion to a "freshwater type" lipid composition, suggesting that lipid metabolism could be modified in response to ambient salinity changes.

A number of studies have also observed a significantly elevated level of arachidonic acid (20:4n-6; AA) in tissues of wild parr and smolts compared to their cultured counterparts (13–15). The cultured parr and smolts all contained considerable amounts of dietary-derived 18:2n-6 which was not desaturated and elongated to AA and led to the suggestion by Ackman and Takeuchi (13) that these reactions might not be possible in salmon undergoing smoltification. The importance of long-chain PUFA in adaptation to salinity changes has been observed in essential fatty acid (EFA) deficient trout, which had reduced ionic permeability in brush border membranes (16) while a similar EFA-deficiency caused morphological changes in the gills of turbot (17). In addition, membrane fatty acid composition is known to affect the activity of a number of ion pumps and membrane-bound enzymes, including Na^+,K^+-ATPase (18,19). The C_{20} PUFA are substrates for eicosanoid synthesis, and AA is the preferred substrate in fish, despite EPA being more abundant in fish phospholipids (20,21). Prostaglandins are known to mediate fluid and electrolyte fluxes in fish gill and kidney and are therefore important in adaptation to changes in salinity (22,23).

In the present study, duplicate groups of Atlantic salmon parr were fed either a control diet containing a Northern hemisphere fish oil, similar to that in commercial feeds used in the salmon farming industry, or a blend of rapeseed and linseed oils providing a fatty acid composition more closely resembling that of freshwater invertebrates. Fish were sampled on a regular basis from 6 mon before seawater transfer to 4 mon after seawater transfer. At each sampling time, fatty acyl desaturation and elongation activity were determined in isolated hepatocytes and prostaglandin F isomers, derived from both AA and EPA, were measured in isolated gill cells stimulated with the calcium ionophore A23187. Samples of liver and gill were also collected for lipid compositional analysis. Seawater tolerance tests were performed on the fish in the weeks preceding seawater transfer.

MATERIALS AND METHODS

Animals and diets. Atlantic salmon parr (1,160) supplied by Marine Harvest McConnell Ltd. (MHM) (Highland, Scotland) were distributed randomly into four 2 m × 2 m tanks at the MHM smolt cultivation unit in Invergarry, Invernesshire, Scotland, in October 1994. Each tank contained 1520 L and was supplied with freshwater at a rate of 45 L/min. The fish oil-containing diet (FO) and the vegetable oil-containing diet (VO) were fed to satiation, to duplicate tanks of fish, using automatic feeders. Experimental diets were prepared by BOCM-Pauls (Renfrew, Scotland). The composition of the diets is shown in Table 1. The freshwater diets (FO and VO) contained 54% protein and 19% lipid while the posttransfer diet contained 49% protein and 24% lipid. The fatty acid compositions of the diets are shown in Table 2. All diets were formulated to satisfy the nutritional requirements of salmonid fish (24).

In late April, 240 fish which had undergone smoltification (i.e., those which had changed to silver coloration) were transferred to the MHM research unit at Inverailort, Invernesshire, Scotland. Fish were distributed randomly into four 1 m × 1 m tanks of 250 L capacity. Fish were initially acclimated in freshwater for a few days before being switched to seawater, supplied at 6 L/min. All fish were then switched to the posttransfer diet which was fed to satiation by automatic feeders. At the outset of the experiment in October, the water temperature was 10.8°C which fell to 0.7°C in January after which the freshwater temperature rose slowly to 5.5°C in April. On transfer to seawater in early May (week 26), the temperature was 9°C and increased to a maximum of 14°C in August. Six fish per dietary treatment were sampled at regular intervals between October 1994 and August 1995.

Lipid extraction and phospholipid fatty acid analysis. Intact gill arches and liver were dissected from three fish per dietary treatment at each time point and immediately frozen in liquid nitrogen. Samples were stored at −20°C prior to lipid extraction. Total lipid was extracted from gill, liver, and diet samples by the method of Folch *et al.* (25). Total polar lipid (PL) fraction was prepared by separating neutral lipids from PL by thin-layer chromatography (TLC) using hexane/diethylether/acetic acid (80:20:2, by vol) as developing solvent. The PL retained on the origin was scraped and subjected to acid-catalyzed transesterification as described by Christie (26). The fatty acid methyl esters were separated and quanti-

TABLE 1
Feed Components (% of dry diet)

Component	FO diet	VO diet	Posttransfer diet
Fish meal[a]	55	29	65
Plant meals[b]	27.5	40.5	15
Porcine blood meal	2	14	1
Micronutrients[c]	2.5	2.5	2.5
Northern hemisphere fish oil	13	—	16.5
Rapeseed/linseed oil (1:1,vol/vol)	—	14	—

[a]Low-temperature fish meal (Aquaculture grade); principally derived from herring and capelin.
[b]Soybean meal and wheat meal.
[c]Vitamin and mineral mixes; VO, vegetable oil; FO, fish oil.

TABLE 2
Fatty Acid Compositions (wt% of total fatty acids) of Diets[a]

	Diet		
Fatty acid	FO diet	VO diet	Posttransfer
14:0	5.1 ± 0.1[a]	1.0 ± 0.0[b]	5.3 ± 0.3[a]
16:0	14.7 ± 0.1[a]	8.3 ± 0.2[b]	13.3 ± 0.2[c]
18:0	2.6 ± 0.1	2.6 ± 0.4	2.4 ± 0.1
Total saturated[b]	23.4 ± 0.2[a]	12.5 ± 0.6[b]	21.9 ± 0.6[c]
16:1n-7	5.3 ± 0.1[a]	1.3 ± 0.1[b]	4.9 ± 0.3[a]
18:1n-9	13.0 ± 0.1[a]	29.1 ± 0.8[b]	12.5 ± 0.2[a]
18:1n-7	2.7 ± 0.3[a]	2.1 ± 0.1[b]	2.6 ± 0.1[a]
20:1n-9	7.9 ± 0.6[a]	2.0 ± 0.3[b]	9.2 ± 0.4[c]
22:1n-11	12.0 ± 0.2[a]	1.6 ± 0.2[b]	12.5 ± 0.2[a]
24:1	0.8 ± 0.1[a]	0.3 ± 0.1[b]	0.9 ± 0.1[a]
Total monounsaturated[c]	42.5 ± 0.3[a]	36.7 ± 1.0[b]	43.6 ± 1.0[a]
18:2n-6	5.6 ± 0.1[a]	21.6 ± 0.3[b]	2.9 ± 0.2[c]
20:2n-6	0.3 ± 0.0[a]	trace[b]	0.2 ± 0.1[a]
20:4n-6	0.6 ± 0.0[a]	0.2 ± 0.0[b]	0.6 ± 0.0[a]
Total n-6 PUFA[d]	6.7 ± 0.1[a]	21.8 ± 0.3[b]	4.2 ± 0.3[c]
18:3n-3	1.7 ± 0.1[a]	23.5 ± 0.2[b]	2.2 ± 0.7[a]
18:4n-3	2.7 ± 0.0[a]	0.4 ± 0.1[b]	2.9 ± 0.1[a]
20:4n-3	0.8 ± 0.0[a]	0.1 ± 0.0[b]	0.9 ± 0.1[a]
20:5n-3	7.3 ± 0.1[a]	1.7 ± 0.1[b]	6.6 ± 0.3[c]
22:5n-3	1.2 ± 0.1[a]	0.3 ± 0.0[b]	1.3 ± 0.2[a]
22:6n-3	10.8 ± 0.1[a]	2.2 ± 0.1[b]	10.7 ± 1.1[a]
Total n-3 PUFA	24.4 ± 0.1[a]	28.2 ± 0.3[b]	24.6 ± 1.1[b]
Total PUFA	31.1 ± 0.1[a]	50.0 ± 0.5[b]	28.8 ± 1.3[a]
n-3/n-6	3.6 ± 0.0[a]	1.3 ± 0.0[b]	5.9 ± 0.1[c]
20:4n-6/20:5n-3	0.08 ± 0.01[a]	0.12 ± 0.01[b]	0.09 ± 0.01[a]
22:6n-3/20:5n-3	1.47 ± 0.02[a]	1.34 ± 0.05[b]	1.62 ± 0.12[a]

[a]Results are means ± SD ($n = 3$); trace, <0.05%; PUFA, polyunsaturated fatty acids. Values in the same row having different superscript letters are significantly different ($P < 0.05$).
[b]Totals include 15:0, 17:0, and 20:0.
[c]Totals include 16:1n-9 and 20:1n-7.
[d]Totals include 18:3n-6, 20:3n-6, and 22:5n-6. See Table 1 for other abbreviations.

fied by gas–liquid chromatography (Carlo Erba Vega 8160; Fisons Ltd., Crawley, United Kingdom) using a 30 m × 0.32 mm capillary column (CP wax 52CB; Chrompak Ltd., London, United Kingdom). Hydrogen was used as carrier gas, and temperature programming was from 50 to 150°C at 40°C/min and then to 230°C at 2.5°C/min. Individual methyl esters were identified by comparison to known standards and by reference to published data (27).

Preparation of isolated hepatocytes and gill cells. Fish were killed by a blow to the head and the liver and gill arches dissected immediately. The gall bladder was removed carefully from the liver and the main blood vessels removed. The liver was perfused *via* the hepatic vein with solution A [calcium- and magnesium-free Hank's balanced salt solution (HBSS) + 10 mM HEPES + 1 mM EDTA] to clear blood from the tissue. The liver was chopped finely with scissors and incubated with 20 mL of solution A containing 0.1% (wt/vol) collagenase in a 25-mL "Reacti-flask" in a shaking water bath at 20°C for 45 min. The digested liver was filtered through 100 μm nylon gauze and the cells collected by centrifugation at 1000 × *g* for 5 min. The cell pellet was washed with 20 mL of solution A containing 1% wt/vol fatty acid-free bovine serum albumin (FAF-BSA) and recentrifuged. The hepatocytes were resuspended in 10 mL of Medium 199 containing 10 mM HEPES, 2 mM glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. One hundred μL of cell suspension was mixed with 400 μL of Trypan Blue, and hepatocytes were counted and their viability assessed using a hemocytometer. One hundred μL of the cell suspension was retained for protein determination.

The gills were blotted on tissue paper to remove blood clots and water after which they were chopped with scissors and incubated in 30 mL solution A containing 0.1% collagenase as described above. Gill cells were prepared as described previously (1) and were resuspended finally in 1 mL of HBSS containing 2 mM $CaCl_2$. Fifty μL were removed for protein determination.

Assay of hepatocyte fatty acyl desaturation/elongation activities. Five mL of each hepatocyte suspension were dispensed into two 25 cm^2 tissue culture flasks. Hepatocytes were incubated with 0.25 μCi of either [1-^{14}C] 18:3n-3 or [1-^{14}C] 18:2n-6, added as complexes with FAF-BSA. Briefly, 25 μCi of fatty acid (0.5 μmol) in ethanol were placed in a reaction vial, solvent evaporated under a stream of nitrogen and 100 μL of 0.1M KOH added. The mixture was stirred for 10 min at room temperature before 5 mL of 50 mg/mL FAF–BSA in HBSS containing 10 mM HEPES buffer were added and the mixture stirred for 45 min at 20°C. After addition of isotope, the flasks were incubated at 20°C for 3 h. After incubation, the cell layer was dislodged by gentle rocking, the cell suspension transferred to conical glass test tubes, and the flasks washed with 1 mL of ice-cold HBSS containing 1% FAF-BSA. The cell suspensions were centrifuged at 300 g for 2 min, the supernatant discarded, and the cell pellets washed with 5 mL of ice-cold HBSS/FAF-BSA. The supernatant was discarded, and the tubes placed upside down on paper towels to blot for 15 s before extraction of total lipid using ice-cold chloroform/methanol (2:1, vol/vol) containing 0.01% (wt/vol) butylated hydroxytoluene (BHT) essentially as described by Folch *et al.* (25) and as described in detail previously (28).

Total lipid was transmethylated, and fatty acid methyl esters prepared as described above. The methyl esters were redissolved in 100 μL hexane containing 0.01% BHT and applied as 2.5 cm streaks to TLC plates impregnated by spraying with 2 g silver nitrate in 20 mL acetonitrile and preactivated at 110°C for 30 min. Plates were fully developed in toluene/acetonitrile (95:5, vol/vol) (29). Autoradiography was performed with Konica A2 film for 4–7 d at room temperature. Silica corresponding to individual PUFA was scraped into scintillation minivials containing 2.5 mL of scintillation fluid (Ecoscint A, National Diagnostics, Atlanta, GA) and radioactivity determined in a TRI-CARB 2000CA scintillation counter (United Technologies Packard, Pangbourne,

United Kingdom). Results were corrected for counting efficiency and quenching of ^{14}C under exactly these conditions.

Ionophore stimulation and eicosanoid extraction. Gill cell suspensions were incubated at 20°C in a shaking water bath and stimulated with A23187 (2 µL in dimethylsulfoxide, 10 µM final concentration) as described previously (1). The incubation was continued for 20 min after which the cells were removed by centrifugation (12,000 × *g*, 2 min) and the supernatant transfered to tubes containing 150 µL ethanol and 50 µL 2 M formic acid. Samples were frozen immediately in liquid nitrogen and stored at −20°C until extraction and measurement of eicosanoids. The frozen gill cell supernatants were thawed and centrifuged (12,000 × *g*, 2 min) to remove any precipitate. The supernatants were extracted using octadecyl silica (C18) Sep-Pak minicolumns (Millipore Ltd., Watford, United Kingdom) by the method of Powell (30) and as described in detail by Bell *et al.* (21). The final extract was dissolved in 0.1 mL of methanol prior to high-performance liquid chromatography (HPLC).

Separation and measurement of prostaglandin F (PGF) isomers. The PGF isomers were separated by reverse-phase HPLC largely as described in Bell *et al.* (21). The PGF isomers were separated using a Spherisorb 5 µm (ODS 2) column (25 cm × 4.6 mm i.d.; Anachem, Luton, United Kingdom). An isocratic solvent system was employed containing 17 mM phosphoric acid/acetonitrile (70:30, vol/vol) at a flow rate of 1 mL/min. Fractions corresponding to each PGF isomer were pooled and extracted as described previously (21). Measurement of PGF isomers of the 2- and 3-series was performed using an enzyme immunoassay kit for $PGF_{2\alpha}$ according to the manufacturer's protocols (SPI-Bio, Massy, France). Cross-reactivity of $PGF_{3\alpha}$ with the $PGF_{2\alpha}$ antibody was 100%.

Seawater challenge and plasma chloride determination. In late March, while still in freshwater, 20 fish per dietary treatment were subjected to a seawater challenge test to assess their ability to osmoregulate. The fish were transferred to a tank containing standardized seawater at a salinity of 35 parts per thousand in a static tank, with aeration, for a period of 24 h. Artificial seawater was produced by adding the appropriate volume of "Seamix" sea salts (Peacock Salt Ltd., Glasgow, Scotland) to freshwater. After 24 h, the fish were euthanized and blood removed *via* the caudal vein into heparinized tubes. Tubes were mixed, centrifuged immediately, and the plasma removed and stored at −20°C. Plasma chloride concentrations were measured using a Jenway Chloride meter (Model PCLM3; Fisons, Loughborough, United Kingdom) using the method described in the operating manual. Plasma chloride concentrations were measured again in early May following transfer to natural seawater at a salinity concentration of 33 parts per thousand.

Protein determination. Protein concentration in isolated gill cell and hepatocyte suspensions was determined according to the method of Lowry *et al.* (31) after incubation with 0.25 mL of 0.25% (wt/vol) SDS/1M NaOH for 45 min at 60°C.

Materials. [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6 (both 50–55 mCi/mmol) were from NEN [DuPont (United Kingdom) Ltd., Stevenage, United Kingdom]. HBSS, Medium 199, HEPES buffer, glutamine, penicillin, streptomycin, collagenase (type IV), FAF-BSA, calcium ionophore A23187, BHT, and silver nitrate were obtained from Sigma Chemical Co. (Poole, United Kingdom). TLC plates, precoated with silica gel 60 (without fluorescent indicator) were obtained from Merck (Darmstadt, Germany). $PGF_{3\alpha}$ and $PGF_{2\alpha}$, both >98%, were obtained from SPI-bio. All solvents were HPLC grade and were obtained from BS & S Ltd. (Edinburgh, Scotland).

Statistical analysis. Significance of difference ($P < 0.05$) between observations at each time point, for each dietary treatment, was determined by analysis of variance using a Statgraphics (system 3.0) computer package (Statistical Graphics Corporation, Rockville, MD). Significance of difference between dietary treatments at each time point was determined by Student's *t*-test. Data which were identified as nonhomogeneous (Bartlett's test) were subjected to either arcsine, square root, or log transformation before analysis. Differences between means were determined by Tukey's test.

RESULTS

All fish grew well on both the FO and VO diets and there were no significant differences in growth rates between dietary treatments, during either the freshwater or seawater stages of the experiment (Table 3). The total C_{18} PUFA substrate further desaturated (and elongated) to tri-, tetra-, penta-, and hexaene products are shown in Figure 1. The metabolites of linolenic acid (18:3n-3) are shown in Figure 1A, and these were always around fivefold higher than metabolites derived from linoleic acid (18:2n-6) shown in Figure 1B. Fatty acid desaturation activity increased significantly, with both PUFA, and in fish fed both FO and VO, up to the time of seawater transfer (26 wk) after which desaturation/elongation was reduced. Desaturation/elongation activities with 18:3n-3 were significantly greater in fish fed VO, compared to fish fed FO, at 7, 22, and 26 wk, while with 18:2n-6 desaturation/elongation was significantly increased in fish fed VO only at 22 and 26 wk.

The levels of AA, EPA, and DHA in gill PL are shown in Figure 2. AA in the gill PL of fish fed VO increased significantly up to 22 wk, before decreasing immediately after sea-

TABLE 3
Growth Parameters During the 45-wk Experimental Period[a]

Parameter	FO diet	VO diet
Number of fish	580	580
Initial weight (g)	24.4 ± 4.7	21.9 ± 4.7
Weight (g) (week 25)[b]	36.8 ± 6.8	34.7 ± 7.2
Final weight (g)	122.5 ± 33.3	118.8 ± 24.4
Specific growth rate[c]	0.51	0.54

[a]Values for initial and final weights are mean ± SD.
[b]Mean weight ± SD immediately before seawater transfer.
[c]Specific growth rate calculated as percentage weight gain/day = $(e^{G_w} - 1) \times 100$, where G_w (daily growth rate) = $\ln W_1 - \ln W_0/T$ (W_1 = final weight, W_0 = initial weight, and T = time in days). See Table 1 for abbreviations.

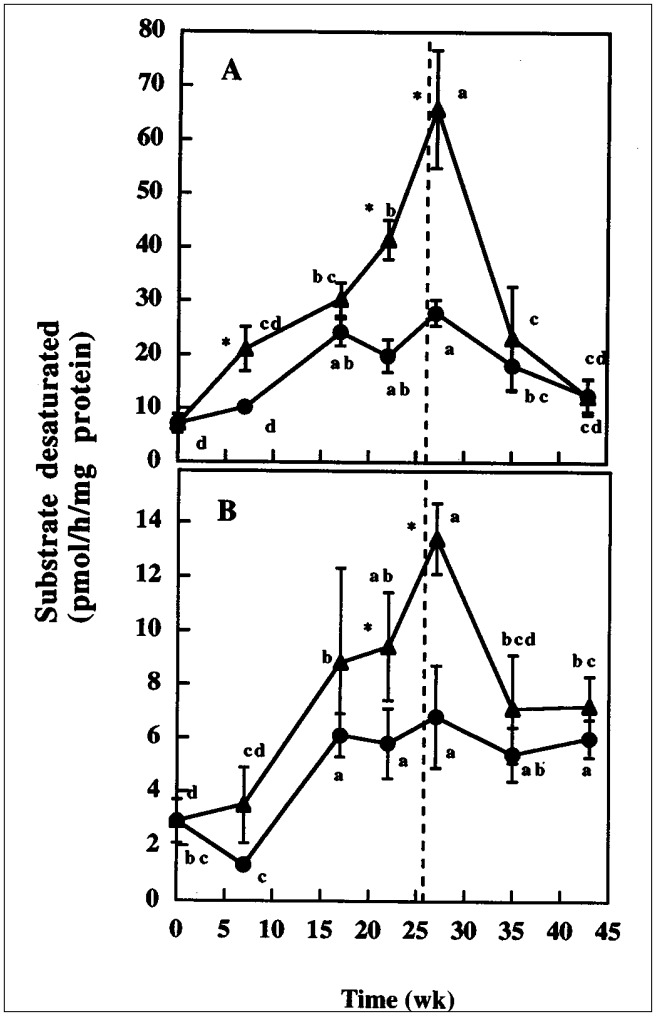

FIG. 1. Total amount of fatty acid desaturated and elongated/h/mg protein by isolated hepatocytes from Atlantic salmon juveniles fed diets containing either fish oil (FO) (●) or vegetable oil (VO) (▲). A, 18:3n-3; B, 18:2n-6. The vertical dotted line represents the time of seawater transfer (26 weeks). Each point represents the mean ± SD for three replicate samples. Points within the same dietary treatment having a different assigned letter are significantly different ($P < 0.05$). Points at the same sampling time assigned an asterisk (*) are significantly different between dietary treatments.

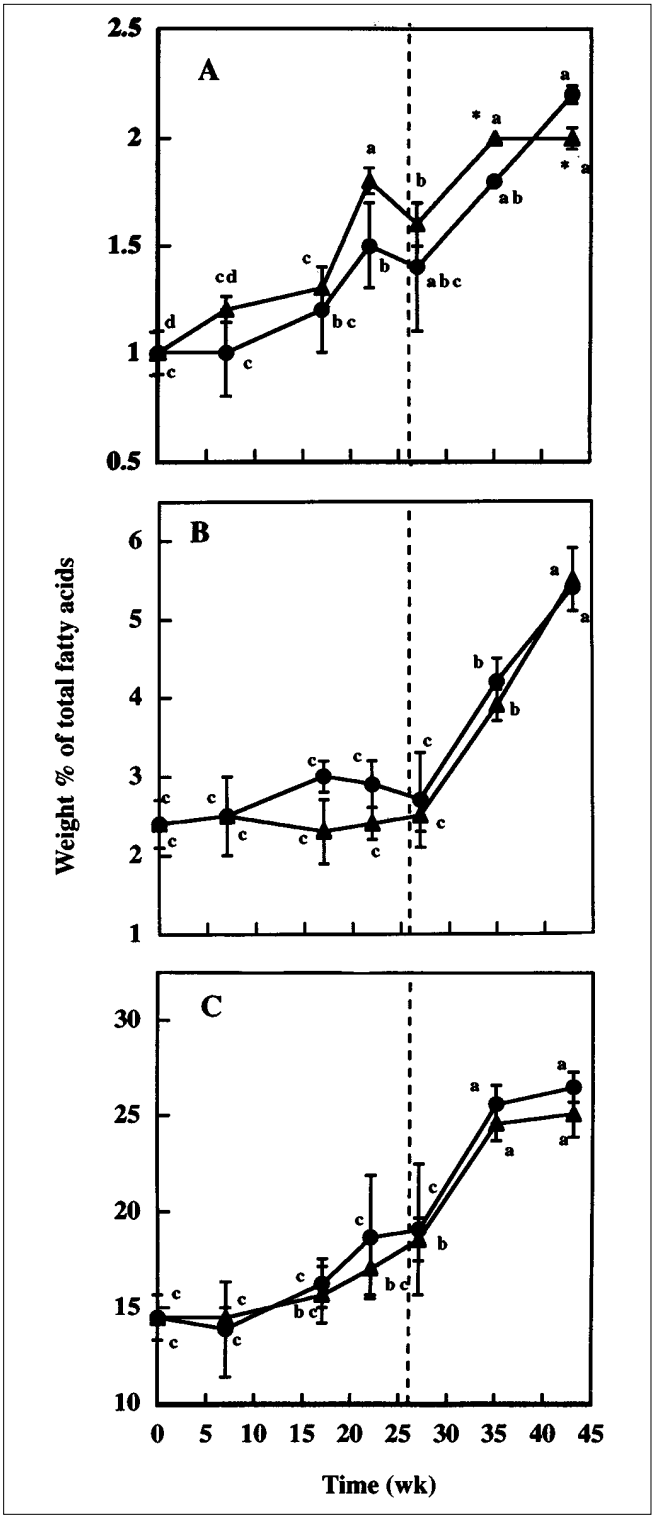

FIG. 2. Levels of the highly unsaturated fatty acids (20:4n-6, A; 20:5n-3, B and 22:6n-3, C) in polar lipids of gill from Atlantic salmon juveniles fed diets containing either FO (●) or VO (▲). The vertical dotted line represents the time of seawater transfer (26 wk).Values are percentage weight of total fatty acids and each point represents the mean ± SD for three replicate samples. Points within the same dietary treatment having a different assigned letter are significantly different ($P < 0.05$). Points at the same sampling time assigned an asterisk (*) are significantly different between dietary treatments. See Figure 1 for abbreviations.

water transfer, and thereafter returning to the pre-seawater transfer level (Fig. 2A). AA in gill PL of fish fed FO followed a very similar pattern to those fed VO although AA was significantly increased at 43 wk compared to the value immediately before seawater transfer (22 wk) (Fig. 2A). EPA in the gill PL of fish fed both diets remained constant in the period up to seawater transfer but increased significantly following seawater transfer (Fig. 2B). DHA in gill PL increased slightly, in fish fed both diets, during the period up to seawater transfer before rising significantly between 26 and 35 wk before leveling off thereafter (Fig. 2C).

The levels of AA, EPA, and DHA in liver PL are shown in Figure 3. The mean AA levels in liver PL of fish fed VO were

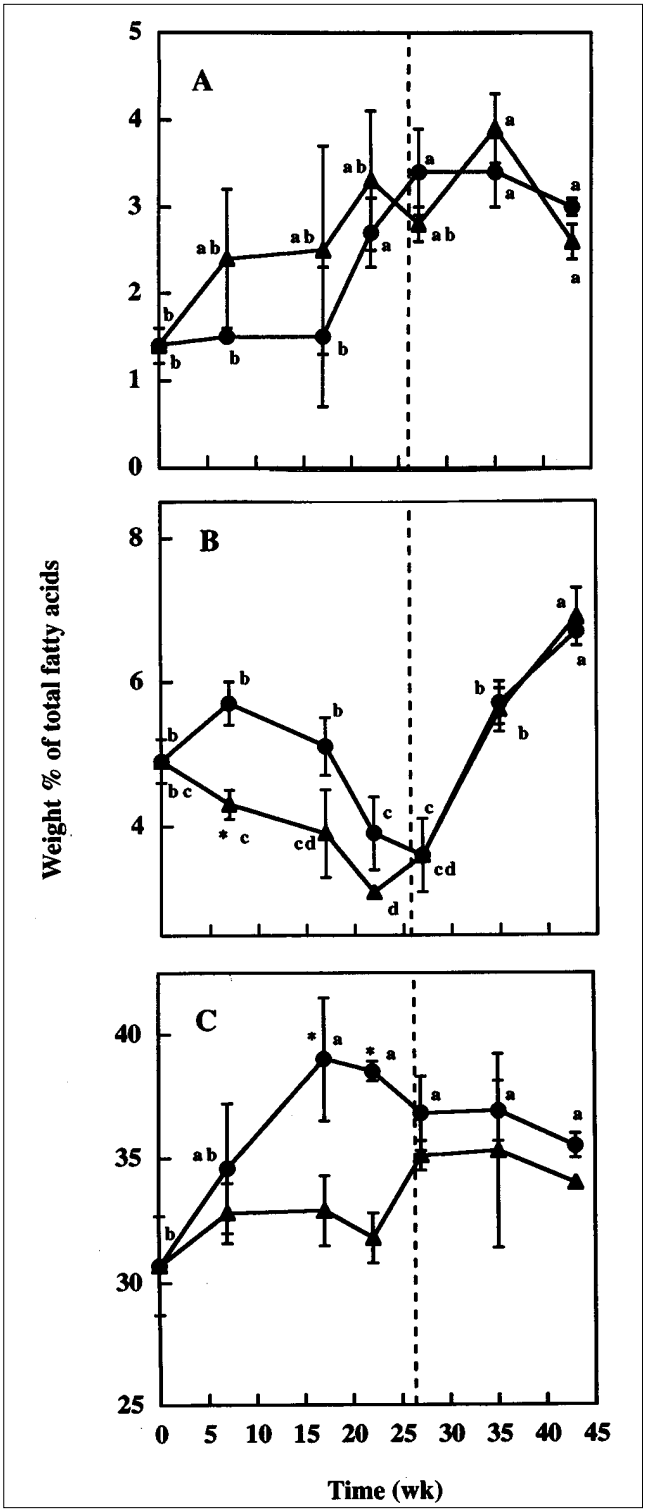

FIG. 3. Levels of the highly unsaturated fatty acids (20:4n-6, A; 20:5n-3, B and 22:6n-3, C) in polar lipids of liver from Atlantic salmon juveniles fed diets containing either FO (●) or VO (▲). The vertical dotted line represents the time of seawater transfer (26 wk).Values are percentage weight of total fatty acids, and each point represents the mean ± SD for three replicate samples. Points within the same dietary treatment having a different assigned letter are significantly different ($P < 0.05$). Points at the same sampling time assigned an asterisk (*) are significantly different between dietary treatments. See Figure 1 for abbreviations.

increased significantly at 35 and 43 wk compared to week 0 (Fig. 3A). AA in liver PL of fish fed FO remained constant until 17 wk before increasing significantly toward seawater transfer and leveling off thereafter (Fig. 3A). EPA levels in liver PL of fish fed VO decreased significantly between week 0 and week 22 before rising slightly at 26 wk and increasing rapidly and significantly thereafter (Fig. 3B). EPA in liver PL of fish fed FO showed a similar trend although levels of EPA were always greater in the freshwater phase, significantly so at 7 wk, compared to fish fed VO (Fig. 3B). DHA levels in liver PL of fish fed VO remained constant during the freshwater phase before increasing, although not significantly, following seawater transfer (Fig. 3C). DHA levels in liver PL of fish fed FO increased significantly from week 0 to week 17 before leveling off in the period immediately pre- and post-seawater transfer. DHA levels were significantly increased in liver PL of fish fed FO at weeks 17 and 22 compared to fish fed VO (Fig. 3C).

The AA/EPA ratios in gill and liver PL are shown in Figure 4. In gill polar lipid from fish fed VO, the AA/EPA ratio increased significantly between week 0 and week 22 before decreasing at seawater transfer and reducing to the initial value (week 0) by week 43 (Fig. 4A). In gill PL from fish fed FO, the AA/EPA ratio remained constant from week 0 to week 17 before increasing slightly, but significantly, at seawater transfer and then reducing to the initial value during the seawater ongrowing period. The AA/EPA ratio in gill PL was significantly increased in fish fed VO at weeks 17, 22, and 35 compared to fish fed FO (Fig. 4A). In liver PL, the changes in AA/EPA ratio due to smoltification and diet were qualitatively similar to those described in gill, although there were no significant differences between dietary treatments. (Fig. 4B).

The production of prostaglandins $PGF_{2\alpha}$ and $PGF_{3\alpha}$ by isolated gill cells stimulated with the calcium ionophore A23187 is shown in Figure 5. The levels of $PGF_{2\alpha}$ produced by stimulated gill cells were always in excess (2- to 4-fold) of $PGF_{3\alpha}$. The levels of $PGF_{2\alpha}$ in fish fed VO increased significantly between 0 and 7 wk before decreasing toward seawater transfer. After seawater transfer, $PGF_{2\alpha}$ levels increased in fish fed VO to reach a peak value at 35 wk (Fig. 4A). The levels of $PGF_{2\alpha}$ in fish fed FO were not significantly different during the trial period. Production of $PGF_{2\alpha}$ was significantly greater at week 17 and week 35 in fish fed VO compared to fish fed FO (Fig. 4A). The changes in $PGF_{3\alpha}$ production in stimulated gill cells during the experimental time course followed a broadly similar pattern, for both dietary treatments, to that described for $PGF_{2\alpha}$ (Fig. 4B). Production of $PGF_{3\alpha}$ was significantly greater at weeks 17, 35, and 43 in fish fed VO compared to fish fed FO (Fig. 4B).

The plasma chloride concentrations in fish subjected to seawater challenge at week 20 are shown in Figure 6. Regression analysis of the plasma chloride concentrations for fish fed VO (dotted line), compared to those for fish fed FO (solid line), indicated that the latter group exhibited higher plasma chloride concentrations, suggesting the FO-fed fish were less able to osmoregulate compared to those fed VO. The mean

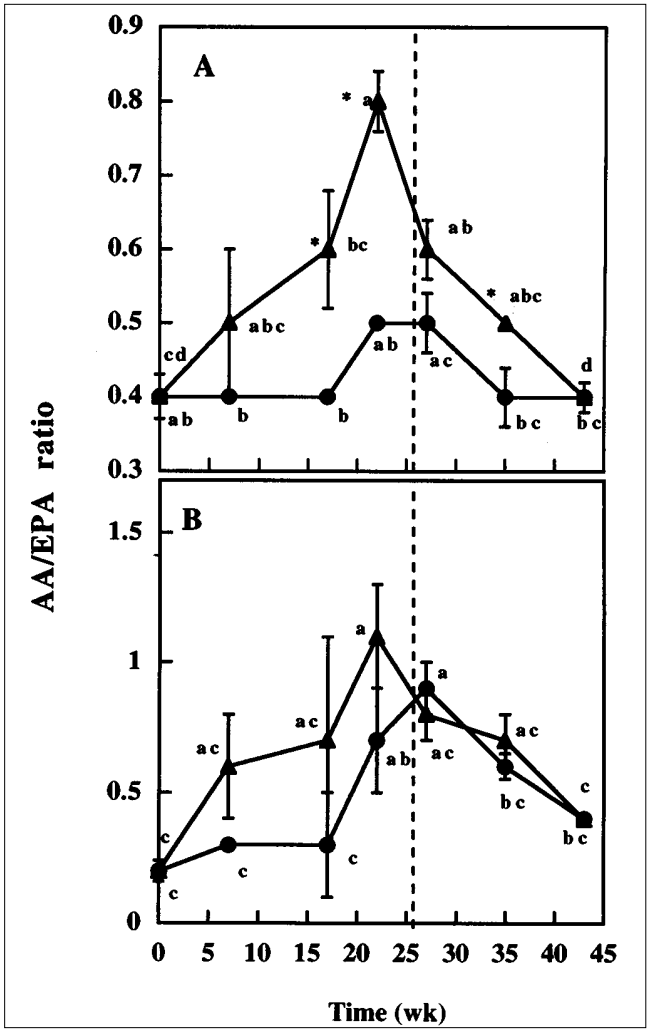

FIG. 4. The ratio of arachidonic acid 20:4n-6/eicosapentaenoic acid 20:5n-3 (AA/EPA) in the polar lipids of gill (A) and liver (B) from Atlantic salmon juveniles fed diets containing either FO (●) or VO (▲). The vertical dotted line represents the time of seawater transfer (26 wk). Each point represents the mean ± SD for three replicate samples. Points within the same dietary treatment having a different assigned letter are significantly different ($P < 0.05$). Points at the same sampling time assigned an asterisk (*) are significantly different between dietary treatments. See Figure 1 for other abbreviations.

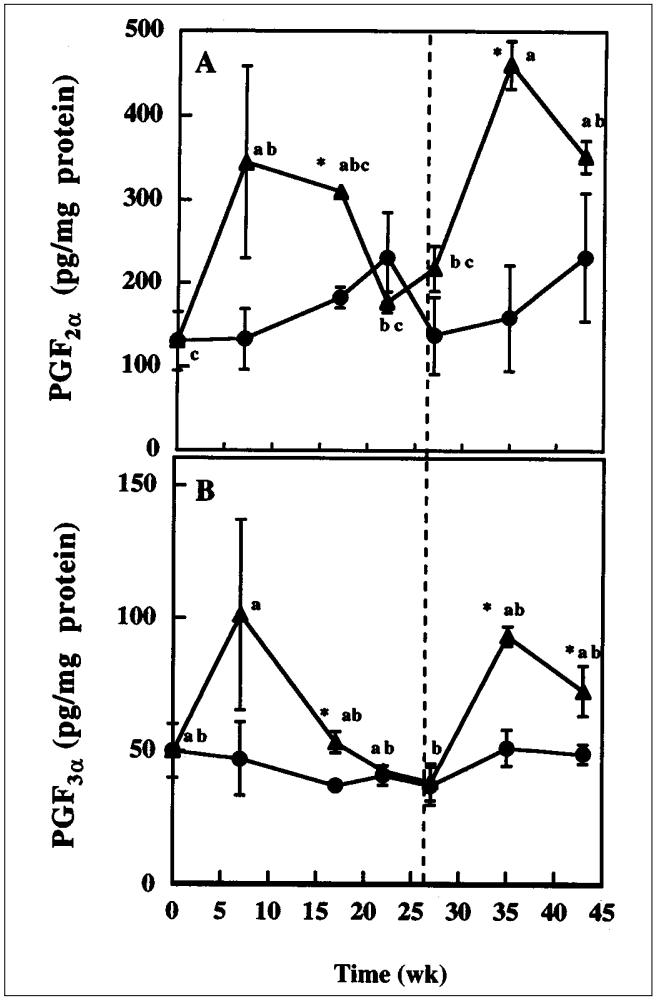

FIG. 5. Production of prostaglandin F analogues by isolated gill cells stimulated with the calcium ionophore A23187. Concentrations of prostaglandin F ($PGF_{2\alpha}$) (A) and $PGF_{3\alpha}$ (B) in pg/mg protein were measured in Atlantic salmon juveniles fed diets containing either FO (●) or VO (▲). The vertical dotted line represents the time of seawater transfer (26 wk). Each point represents the mean ± SD for three replicate samples. Points within the same dietary treatment having a different assigned letter are significantly different ($P < 0.05$). Points at the same sampling time assigned an asterisk (*) are significantly different between dietary treatments. See Figure 1 for other abbreviations.

values for plasma chloride concentration were 149.6 ± 7.4 and 163.2 ± 15.4 mM for fish fed VO and FO, respectively (significantly different, $P < 0.05$). The seawater tolerance test was repeated at week 26, 24 hr after transfer to seawater of 33 parts per thousand. The plasma chloride values recorded at that time were 133.4 ± 5.1 and 132.1 ± 6.3 mM for fish fed VO and FO, respectively.

DISCUSSION

The present study provides evidence that fatty acyl desaturation and elongation in Atlantic salmon hepatocytes is determined by an interplay between the genetic and environmental actions which constitute the smoltification or parr-smolt transformation processes and by dietary fatty acid composition. Fish fed FO showed increased desaturation activity, with both C_{18} substrate fatty acids, up to seawater transfer, with a subsequent reduction in activity posttransfer. Fish fed VO showed the same pattern although activities were higher (up to 3-fold) than in the FO fish, presumably at least partly owing to the greater supply of substrate fatty acids in the VO diet. At all times during the experimental period, 18:3n-3 was preferred over 18:2n-6 as the precursor in the desaturation and elongation pathways, the former being utilized at a rate around 5-fold higher than the latter. This observation is in agreement with previous studies in rats (32), rainbow trout

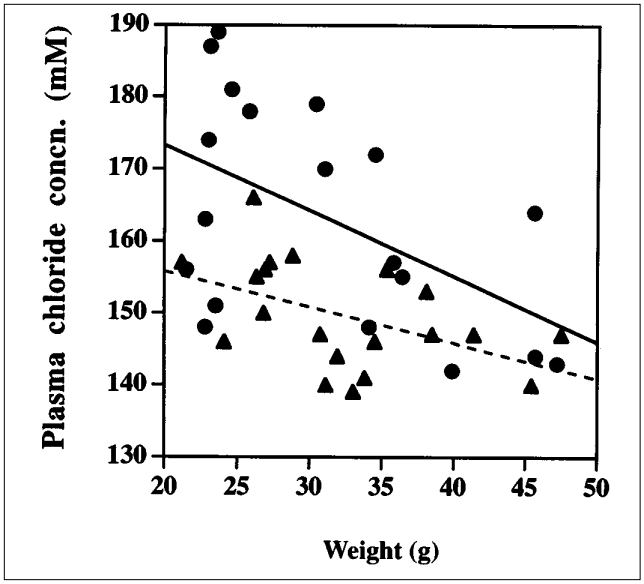

FIG. 6. Plasma chloride concentration (concn.) (mM) vs. weight (g) in Atlantic salmon parr fed diets containing either FO (●) or VO (▲) after being subjected to a seawater challenge at 20 wk as described in the Materials and Methods section. The regression lines for fish fed FO (—) or VO (---) are indicated. See Figure 1 for abbreviations.

(33), and established fish cell lines (34). Fish oils rich in long-chain n-3 PUFA are inhibitory to desaturation of both 18:3n-3 and 18:2n-6 in rats (35,36) and rainbow trout (37). The results of the present study in Atlantic salmon are also consistent with this and explain the apparent absence of 18:2n-6 desaturation products in farmed salmon, which were present in wild fish observed in a previous investigation (13). Given that most aquatic insects are rich in 18:2n-6 and 18:3n-3, it seems likely that the pattern of fatty acyl desaturation activity occurring during smoltification in wild fish would more closely resemble that found in fish fed VO compared to those fed FO.

It has been long established that marine lipids contain more PUFA, particularly long-chain n-3 PUFA, resulting in a high n-3/n-6 PUFA ratio compared to freshwater lipids (38). While this difference partly reflects the input from the respective food chains, it is also influenced by the modification of ingested dietary lipids *via* desaturation and elongation (5). The change in lipid composition from a "freshwater type" to a "marine type" has been observed in other salmonid species such as masu salmon (*O. masou)* and coho salmon *(O. kisutch)* (12,39), and these changes were regarded as a preadaptive response to the marine environment (11). During the course of this study, fish were subject to increasing water temperatures in the period leading up to, and beyond, seawater transfer. While temperature is known to affect PUFA metabolism and membrane phospholipid fatty acid compositions (40), all treatment groups were subject to the same temperature profile, and any differences between dietary treatments were independent of water temperature. The present study confirms that this preadaptation begins up to 6 mon prior to seawater migration and involves a significant "switch-on" of desaturation and elongation activity in hepatocytes.

While a number of studies have measured fatty acid compositions in salmonid parrs and smolts, these have generally involved one observation at each developmental stage (11–14,39). The present study is the first to follow changes in polar lipid fatty acid compositions in the period covering 6 mon prior to seawater transfer until 4 mon posttransfer. In gill polar lipid, DHA increased slowly in the freshwater phase, in both dietary treatments, up to seawater transfer and thereafter increased rapidly during the first 8 wk in seawater before leveling off. However, EPA remained constant in gill polar lipids up to seawater transfer before increasing rapidly in the period following seawater transfer. These results suggest that DHA is increased as a preadaptive response to life in the marine environment which is in agreement with previous observations (11) but also suggest that DHA, and EPA, continue to be incorporated into gill membrane lipid for a considerable period post-seawater transfer. Although the increase in gill DHA postseawater transfer may be due to an increased dietary supply of DHA (the total lipid content of the parr diet was 19% whereas the seawater diet contained 24% lipid), there was no similar increase in DHA in liver PL which suggests that the incorporation into gill has a specific physiological function. There is considerable evidence that the membrane fatty acid composition can influence membrane fluidity and thereby the activities of enzymes located within the membrane (18). More specifically, increased membrane DHA was observed in the gut of seawater-adapted trout (41), in several tissues of seawater-adapted guppies (42), and reduced dietary PUFA, and subsequently reduced membrane DHA, led to destruction of gill epithelia in the turbot (17). A number of studies with mammals have suggested that fish oils rich in EPA and DHA can alter membrane microenvironments and thereby the activity of enzymes such as Na^+, K^+-ATPase (19,43) which has important osmoregulatory functions, especially in fish subjected to changes in salinity.

The levels of AA, in both gill and liver PL, increased by over 100% during the course of the experimental period. In liver, AA levels were increased in fish fed VO compared to fish fed FO in the freshwater phase, while in the same period mean AA values in gill were higher in VO-fed fish compared to fish fed FO. In gill, AA levels continued to rise after seawater transfer suggesting that this n-6 PUFA is important in the smoltification process and in seawater acclimation. AA content of liver PL increased to 2.7 and 3.3% in FO and VO-fed fish, respectively, at week 22. These are comparable to the values of 2.1% observed in liver total lipid of wild salmon parr by Bolgova and Shchurov (14) and of 2.0% in liver phosphatidylcholine by Bell (15).

EPA levels remained virtually constant in gill PL but declined steadily in liver in both dietary treatments up to seawater transfer. This decline may reflect increased synthesis of DHA, required by tissues involved in osmoregulation, during preadaptation to the marine environment. In both gill and

liver, there was a rapid incorporation of EPA into PL in the period following seawater transfer with the level of EPA increasing more than 2-fold after transfer. While this increase may be due to increased dietary intake of EPA in the post-transfer diet as described above, a similar increased incorporation of DHA did not occur in liver and its absence suggests that there is an increased physiological requirement for EPA in post-smolts. It is interesting that, despite the differences in dietary fatty acid composition and fatty acid desaturase activities, the levels of AA, EPA, and DHA appeared to converge at seawater transfer. It would seem that there is a genetically predetermined value for these EFA which must be achieved to allow seawater transition to occur, and that this value was attained despite the differences in dietary fatty acid composition. However, by providing a diet which has a composition more closely resembling that of aquatic insects (6,7), the transitional EFA values may be attained at an earlier stage in the smoltification process and may permit a more successful seawater adaptation.

Both EPA and AA are precursors of biologically active eicosanoids, and the ratio of these two fatty acids varied considerably over the experimental period, especially in gill. In gill PL, the AA/EPA ratio was always greater in fish fed VO compared to those fed FO, with the highest values present immediately before seawater transfer. Production of the AA-derived $PGF_{2\alpha}$ was always in excess (2- to 4-fold) of the EPA-derived $PGF_{3\alpha}$, despite the abundance of the respective precursors being reversed. The preference for AA as an eicosanoid precursor has been observed in a number of fish species, tissues and cultured cells, despite EPA being present in greater abundance in cellular phospholipids (20,21,44). In the present study, both PGF analogues were produced in greater amounts by gill cells from VO-fed fish compared to those fed FO.

The explanation of these results is more complex than merely considering the respective ratios of the precursor PUFA, since eicosanoid production is modulated by a number of physiological and biochemical processes including hormones (23) and phosphospholipase A_2 activity which, in turn, can be influenced by the fatty acid composition of the membrane microenvironment (45,46). Although the physiological functions of eicosanoids have been less well studied in fish compared to mammals, they are involved in reproductive function, hormone release, cardiovascular function, neural function, and osmoregulation (23). Prostaglandins are modulators of ion and electrolyte balance in the kidney (22, 47) and in gills and opercular epithelium (48). In addition, prostaglandins operate synergistically with the hyperosmoregulatory hormone prolactin in the regulation of plasma Na^+ and Ca^{2+} concentrations (49). The studies of Van Praag *et al.* (48) suggest that prostaglandins may affect electrolyte balance at a number of sites including the Na^+/Cl^- co-transporter, the Na^+/K^+-ATPase, the apical chloride conductance, or the basolateral K^+ conductance. In the present study, a seawater challenge performed at 20 wk (6 wk before seawater transfer) showed that fish fed VO had lower concentrations of plasma Cl^- compared to those fed FO. However, when the fish were challenged at 26 wk, immediately after seawater transfer, the plasma Cl^- concentrations were similar for both dietary treatments. It appears that the ability to osmoregulate is related to prostaglandin production and the AA/EPA ratio in tissue PL, and that dietary manipulation can influence the smoltification process.

The present study has provided new information on the important changes in lipid metabolism which are involved in the highly complex physiological process of parr-smolt transformation. The results suggest that a diet utilizing vegetable oils, which more closely mimic the fatty acid composition of freshwater aquatic insects that make up the diet of wild salmon parr, could be more beneficial in effecting the smoltification process than the marine fish oils which are currently favored by the aquaculture industry.

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Modifying the n-3 Fatty Acid Content of the Maternal Diet to Determine the Requirements of the Fetal and Suckling Rat

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ABSTRACT: During perinatal development, docosahexaenoic acid (22:6n-3) accumulates extensively in membrane phospholipids of the nervous system. To evaluate the n-3 fatty acid requirements of fetal and suckling rats, we investigated the accumulation of 22:6n-3 in the brain and liver of pup rats from birth to day 14 postpartum when their dams received increasing amounts of dietary 18:3n-3 (from 5 to 800 mg/100 g diet) during the pregnancy–lactation period. The fatty acid composition of brain and liver phospholipids of pups, as well as that of dam's milk, was determined. At birth, brain 22:6n-3 increased regularly to reach the highest level when the maternal diet contained 800 mg 18:3n-3/100 g. On days 7 and 14 postpartum, brain 22:6n-3 plateaued at a maternal dietary supply of 200 mg/100 g. Docosapentaenoic acid (22:5n-6) had the opposite temporal pattern. The unusually high concentration of eicosapentaenoic acid (20:5n-3) in liver and dam's milk observed at the highest 18:3n-3 intake suggests an excessive dietary supply of this fatty acid. All these data suggest that the n-3 fatty acid requirements of the pregnant rat are around 400 mg 18:3n-3 and those of the lactating rat at 200 mg (i.e., 0.9 and 0.45% of dietary energy, respectively). The values for 18:3n-3 and 22:6n-3 milk content which allowed brain 22:6n-3 to reach a plateau value in suckling pups were 1% of total fatty acids and 0.9% (colostrum) to 0.2% (mature milk), respectively. These levels are similar to those recommended for infant formulas.

Lipids 32, 527–534 (1997).

Docosahexaenoic acid (DHA, 22:6n-3) is present at high concentrations in membrane phospholipids (PL) in the brain and retina, and is deposited primarily during the fetal and early postnatal periods in mammalian species (reviewed in Ref. 1). This suggests that 22:6n-3 plays an essential role in neuronal and visual development. To support this hypothesis, numerous studies have reported that n-3 fatty acid dietary deficiency during development causes functional disturbances in experimental animals, such as impaired learning behaviors and altered electroretinograms in rodents and nonhuman primates, and decreased visual acuity in nonhuman primates (2–4). These effects were related to a depletion of 22:6n-3 in the brain and retina PL (2,3,5). Recent observations of human infants fed a low n-3 fatty acid formula confirmed these relationships (6–8).

Accumulation of 22:6n-3 in the developing brain and retina depends on the dietary intake of n-3 fatty acids, both in the form of the precursor, α-linolenic acid (18:3n-3), and in its long-chain derivatives, eicosapentaenoic acid (20:5n-3) and 22:6n-3 (1). It also depends on the balance between linoleic (18:2n-6) and α-linolenic acids in the diet because of the competition between these two fatty acids for the pathway of desaturation–elongation (1). The precise determination of the nutritional requirements for n-3 fatty acids in humans could be drawn from animal models such as the developing piglet and rat. In comparison with the human species, the perinatal timing of brain growth spurt is very similar in the piglet but is delayed in the rat because the growth starts in the immediate postnatal period (1). The term gestation piglet model is the most suitable model for studying the specific requirements of formula-fed infants, whereas the rat model gives the possibility to easily and precisely assess the dose–effect relationship between the n-3 fatty acid supply of the dam's diet and the level of 22:6n-3 in the offspring (2,9,10). Moreover, the use of n-3-deficient female rats, at the beginning of the experimental design, avoids underestimating the requirements because there is no n-3 fatty acid store (11,12). Yet, the possible pitfall of this protocol can be that the results are affected by the short period of time (2 wk) between n-3 fatty acid refeeding and pregnancy. Previous studies have established that the minimal 18:3n-3 requirement of the developing rat is about 0.4% of dietary energy (i.e., 200 mg/100 g maternal food) with a 18:2n-6 to 18:3n-3 ratio of 6 (2,11). However, the fetal and neonatal requirements were confounded, and the level of n-3 fatty acids in the rat milk was not reported.

The aim of the present study was to determine the individual n-3 fatty acid requirements of the fetal and suckling rat, such as defined in a previous work by the minimal 18:3n-3 content in the maternal diet necessary to reach the plateau (or "optimal") level of 22:6n-3 in the pup brain and liver (2). The level of docosapentaenoic acid (22:5n-6) was also taken into

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Abbreviations: ANOVA, analysis of variance; DHA, docosahexaenoic acid (22:6n-3); FAME, fatty acid methyl ester; PL, phospholipids; TL, total lipids.

account because this fatty acid specifically replaces 22:6n-3 when the dietary n-3 fatty acid supply is insufficient (2,11,12). Data of 1-day-old-pups were used to estimate the 18:3n-3 requirement of the pregnant rat, and that of 3-, 7-, and 14-day-old pups of the lactating rat. The n-3 fatty acid concentration in the dam's milk was determined during the whole period of lactation to provide complementary data on the requirements of the suckling pup.

MATERIALS AND METHODS

Animals and diets. Fifty female Wistar rats from an n-3 fatty acid-deficient lineage (second generation) were reared as previously described (12). They were fed *ad libitum* a semisynthetic diet containing 5% (by weight) peanut oil which supplied about 1 g of 18:2n-6/100 g diet (2.25% of total dietary energy) but a very low 18:3n-3 content (5 mg/100 g diet)(deficient dietary group, Table 1). Two weeks before mating (8 wk of age), they were randomly divided into five dietary groups of $n = 10$ females per group. The first group continued receiving the low 18:3n-3 diet (group A), whereas the four others were fed 5% fat diets containing 100, 200, 400, or 800 mg 18:3n-3/100 g diet (0.22, 0.45, 0.90, or 1.80% total dietary energy, respectively), and designated as groups B, C, D, and E, respectively. The different 18:3n-3 contents were obtained by mixing peanut, rapeseed, or linseed oils as indicated in Table 1. All diets contained about 1 g 18:2n-6/100 g diet because this quantity is considered as the minimum n-6 fatty acid requirements for the brain and other organs in the developing rat (11). Their 18:2n-6 to 18:3n-3 ratios ranged from 1.3 to 10.1.

After parturition, dams were kept in individual plastic cages. In each dietary group, young pups from four different litters were sacrificed by decapitation on days 1, 3, 7, and 14 postpartum. Before sacrifice, all pups were separated from their mothers for 30 min. After a simple observation, pups which had an empty stomach were replaced with their mothers, then allowed to suckle 30 min and immediately sacrificed. With this experimental procedure, the percentage of each fatty acid of total lipids (TL) of the stomach content, and specifically that of medium-chain fatty acids, was similar to that of the milk triacylglycerols as described by Bitman *et al.* (13). The litters were systematically equalized to eight pups and body weights were recorded. The brain, liver, and stomach content (i.e., rat milk) of the pups were removed, weighed, pooled by litter (four pups on days 1 and 3 postpartum and two pups on day 7 postpartum), freeze-dried, and stored at −80°C until analysis.

Lipid analysis. TL of the brain, liver, and stomach content were extracted by the Folch *et al.* (14) procedure in the presence of 0.02% (wt/vol) butylhydroxytoluene. Brain and liver PL were separated using silicic acid cartridges (Supelco France, St. Germain en Laye, France) as described by Juaneda and Rocquelin (15), then transmethylated with 10% BF_3 in

TABLE 1
Oil Content and Fatty Acid Composition of Dietary Lipids[a]

	Dietary group				
	A (deficient)	B	C	D	E
mg 18:3n-3/100 g diet	5	100	200	400	800
Oils (g/100 g diet)					
Peanut	5.00	1.25	2.50	—	—
Rapeseed	—	3.75	2.50	5.00	4.20
Linseed	—	—	—	—	0.80
Total	5.00	5.00	5.00	5.00	5.00
Fatty acid (% total fatty acids)					
16:0	9.4	8.3	7.2	5.4	5.4
18:0	3.1	2.7	2.4	1.5	2.0
18:1n-9	60.2	60.6	60.3	60.6	53.1
18:2n-6	21.0	21.2	21.8	21.9	20.7
18:3n-3	0.1	2.1	4.3	8.3	16.7
20:0	1.3	1.1	0.9	0.6	0.5
20:1n-9	1.1	1.2	1.1	1.1	1.1
22:0	2.6	1.9	1.4	0.3	0.3
24:0	1.2	0.9	0.6	0.3	0.2
Fatty acid	18:2/18:3 210	10.1	5.1	2.7	1.3
(mg/100 g diet)					
18:2n-6	987	996	1025	1029	973
18:3n-3	5	99	202	390	785

[a]The experimental diets contained (g/kg diet): lipids, 50; casein, 220; DL-methionine, 1.6; cellulose, 20; starch, 438.4; saccharose, 220; vitamin mixture, 10; mineral mixture, 40 (caloric density: 3970 Kcal/kg or 16.61 MJ/kg). The vitamin mixture was a total vitamin supplement (United States Biochemical Corp., Cleveland, OH), and the mineral mixture was as follows (g/100 g): $CaHPO_4 \cdot 2H_2O$, 38.0; K_2HPO_4, 24.0; $CaCO_3$, 18.0; NaCl, 6.9; MgO, 2.0; $MgSO_4 \cdot 7H_20$, 9.0; $FeSO_4 \cdot 7H_2O$, 0.86; $ZnSO_4 \cdot H_2O$, 0.5; $MnSO_4 \cdot H_20$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.1; NaF, 0.08; $CrK(SO_4)_2 \cdot 12H_2O$, 0.05; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.002; KI, 0.004; $CoCO_3$, 0.002; Na_2SeO_3, 0.002. Oils were a gift from Lesieur Alimentaire (Boulogne Billancourt, France).

methanol (Fluka, Socolab, Paris, France) (16). Milk TL were hydrolyzed by saponification, methylated in 10% BF_3 methanol, and fatty acid methyl esters (FAME) were extracted with hexane without any evaporation to avoid excessive loss of medium-chain FAME. FAME were analyzed by gas–liquid chromatography and identified as previously reported (17). The fatty acid composition was expressed as a percentage of total fatty acids (wt%).

Statistics. Results are mean ± SD for $n = 4$ values. Differences in the mean levels of fatty acids between the five dietary groups and the four stages of postnatal age were checked using a two-factor analysis of variance (ANOVA) (StatView SE+Graphics™; Abacus Concepts Inc., ASD Meylan, France). At each postnatal age, comparison between the dietary groups was done by one-way ANOVA. Means were compared by Scheffé's test, the level of significance being set at $P < 0.05$. Correlations between milk fatty acids were calculated by simple linear regression analysis.

RESULTS

Brain and liver fatty acid composition at birth. As shown in Table 2 for brain PL, the 18:3n-3 diet content modified only the percentages of n-6 and n-3 fatty acids. The percentages of 22:5n-3 and 22:6n-3 were slightly and considerably increased, respectively, from the dietary group receiving the lowest 18:3n-3 supply (group A) to that receiving the highest 18:3n-3 supply (group E), whereas that of 22:5n-6 had an inverse evolution. The 22:5n-6/22:6n-3 ratio, which is an index of n-3 fatty acid deficiency, was greater than 1 only in brain PL of deficient pups (group A). The percentages of 20:4n-6 and 20:5n-3 were unchanged. In order to obtain a more comprehensive view of the n-3 fatty acid requirements of the fetal rat, the variations of 22:6n-3 and 22:5n-6 contents both in brain and liver PL were reported (Fig. 1). The 22:6n-3 content in brain rose dramatically when the dietary 18:3n-3 was raised from 5 to 200 mg/100 g diet and then rose slowly to reach the highest value at 800 mg (11.1%, $P < 0.05$). No significant difference was noted, however, between dietary levels of 200 and 400 mg (8.7 vs. 9.2%). The 22:5n-6 content had an opposite evolution and reached the lowest value at 800 mg. In liver PL, the 22:6n-3 and 22:5n-6 contents were similarly affected by the quantity of 18:3n-3 in the maternal diet as described for the brain.

Milk fatty acid composition at birth. The percentages of saturated and monounsaturated fatty acids in milk TL at birth

TABLE 2
Fatty Acid (FA) Composition of Brain Phospholipids of One-Day-Old Pups in Relation to the α-Linolenic Content of the Maternal Diet[a]

	Dietary group (% total FA)				
FA	A	B	C	D	E
SFA[b]					
≤12:0	2.1 ± 0.4	1.6 ± 0.5	2.0 ± 0.2	1.4 ± 0.2	1.7 ± 0.5
14:0	2.0 ± 0.1	2.1 ± 0.3	1.9 ± 0.1	2.1 ± 0.2	1.9 ± 0.2
16:0	26.4 ± 1.9	26.7 ± 1.3	26.3 ± 1.3	27.1 ± 0.8	26.7 ± 1.0
18:0	13.7 ± 1.3	13.1 ± 0.5	13.5 ± 0.7	13.3 ± 0.4	13.2 ± 1.3
MUFA[c]					
16:1n-9	9.1 ± 1.2	10.9 ± 1.5	9.0 ± 1.0	10.2 ± 2.0	9.0 ± 1.3
16:1n-7	2.3 ± 0.2	2.7 ± 0.4	2.6 ± 0.3	3.5 ± 1.1	2.6 ± 0.5
18:1n-9	12.8 ± 2.6	11.8 ± 0.3	12.3 ± 0.9	11.9 ± 0.4	12.0 ± 0.5
18:1n-7	5.0 ± 1.6	4.1 ± 1.0	3.9 ± 0.3	3.6 ± 0.4	4.1 ± 0.8
20:1n-9	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20:1n-7	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
n-6 FA					
18:2n-6	0.7 ± 0.2	0.5 ± 0.1	0.8 ± 0.4	0.6 ± 0.1	1.0 ± 0.3
18:3n-6	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
20:2n-6	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
20:3n-6	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.2
20:4n-6	10.0 ± 1.1	10.0 ± 0.8	10.1 ± 0.6	9.8 ± 0.7	10.0 ± 0.5
22:4n-6	2.2 ± 0.3	2.2 ± 0.2	2.2 ± 0.3	2.2 ± 0.1	2.1 ± 0.3
22:5n-6	9.1 ± 1.4[d]	5.4 ± 0.4[e]	3.8 ± 0.4[f]	2.0 ± 0.2[g]	1.2 ± 0.2[h]
n-3 FA					
20:5n-3	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
22:5n-3	0.1 ± 0.0[d]	0.1 ± 0.0[d]	0.4 ± 0.2[d,e]	0.3 ± 0.1[e]	0.7 ± 0.3[f]
22:6n-3	1.5 ± 0.4[d]	5.8 ± 0.6[e]	8.7 ± 0.2[f,g]	9.3 ± 0.7[g]	11.1 ± 1.0[h]
22:5n-6/22:6n-3	6.1 ± 0.8[d]	0.93 ± 0.12[e]	0.44 ± 0.05[f]	0.22 ± 0.06[g]	0.11 ± 0.02[h]

[a]Values are means ± SD ($n = 4$). Means with different superscript (d–h) letters are significantly different ($P < 0.05$) by analysis of variance.
[b]SFA, saturated FA.
[c]MUFA, monounsaturated FA. The sum of minor FA (15:0, 17:0, 20:0, 22:0, 24:0, 20:3n-9, 24:1n-9, 24:4n-6, 24:5n-6, 18:3n-3, 18:4n-3) represents between 0.5 and 1% of total FA and is omitted in the table.

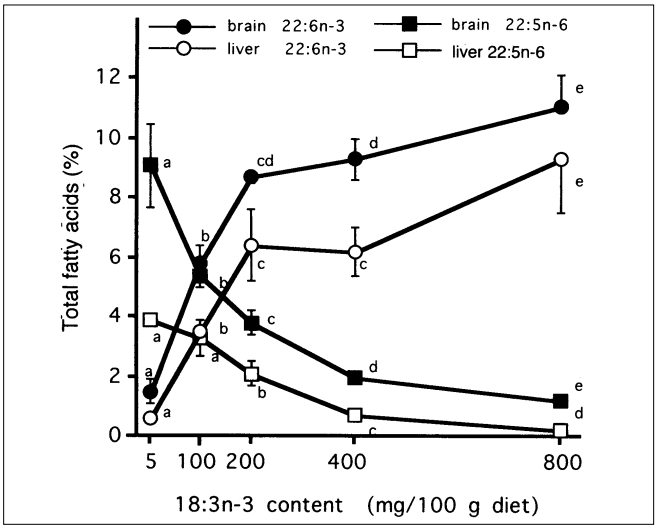

FIG. 1. Effect of the 18:3n-3 content of the maternal diet on the 22:6n-3 and 22:5n-6 contents in brain and liver phospholipids from one-day-old pups. Values are means ± SD ($n = 4$ in each dietary group). Means with different superscripts (a–e) indicate a significant effect of the 18:3n-3 dietary content ($P < 0.05$) by analysis of variance.

(colostrum) were not significantly modified by the 18:3n-3 content of the maternal diet, and accounted for 30–34% and 54–56% of total fatty acids, respectively (Table 3). Changes in n-3 fatty acid reflected the quantity of 18:3n-3 in the maternal diet. The 18:3n-3 content regularly increased from 0% (group A) to 2.4% at the highest dietary intake (group E), as for those of 18:4n-3 and 20:5n-3 (0 to 0.3% and 0 to 0.9%, respectively). For 22:5n-3 and 22:6n-3, a plateau level was reached when the maternal diet supplied 400 mg 18:3n-3/100 g diet (group D). Among n-6 fatty acids, the 22:5n-6 fell regularly to disappear in group E, whereas the 20:4n-6 and 22:4n-6 remained unchanged in the first four dietary groups (2.2–2.5 and 0.9–1.2%, respectively) and then significantly decreased in group E. The 18:2n-6 represented around 5–7% of total fatty acids, and the 18:2n-6/18:3n-3 ratio ranged from 18.3 (group B) to 3.0 (group E), and was systematically 1.7- to 2.4-fold higher than in dietary lipids.

The percentages of 18:3n-3 in colostrum TL, as well as those of n-3 long-chain derivatives (20:5n-3, 22:6n-3), were positively correlated with the dietary 18:3n-3 content ($P < 0.001$) (data not shown).

Long-chain n-3 and n-6 fatty acids in the brain and liver during postnatal development. Figures 2 and 3 illustrate postnatal changes in the percentage of C_{22} (22:6n-3, 22:5n-6) and C_{20} (20:5n-3, 20:4n-6) n-6 and n-3 fatty acids in brain and liver PL according to the dietary intake of 18:3n-3. The 22:6n-3 and 20:4n-6 significantly increased with postnatal age in both the tissues and all the dietary groups ($P < 0.001$), even in the deficient group for 22:6n-3. Conversely, the 20:5n-3 and 22:5n-6 decreased ($P < 0.001$ and $P < 0.04$, respectively), except in the deficient group for 22:5n-6 where it followed a postnatal evolution similar to that of 22:6n-3.

TABLE 3
FA Composition of Total Lipids (TL) of Rat Colostrum (day 1 postpartum) in Relation to the α-Linolenic Content of the Maternal Diet[a]

	Dietary group (% total FA)				
FA	A	B	C	D	E
Total SFA[b]	33.8 ± 2.0	32.4 ± 1.3	33.2 ± 1.9	30.2 ± 1.1	30.4 ± 2.8
Total MUFA[c]	55.3 ± 2.5	55.4 ± 2.0	53.9 ± 2.9	56.2 ± 1.2	53.9 ± 1.8
Total n-6 FA	10.8 ± 0.7	10.9 ± 0.6	10.8 ± 1.1	10.5 ± 1.0	10.3 ± 1.3
18:2n-6	5.0 ± 0.4[d]	5.5 ± 0.7[d,e]	6.0 ± 0.4[d,e]	5.9 ± 0.9[d,e]	7.2 ± 0.9[e]
18:3n-6	0.3 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.2
20:2n-6	0.4 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
20:3n-6	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.0
20:4n-6	2.5 ± 0.3[d]	2.5 ± 0.1[d]	2.2 ± 0.4[d]	2.3 ± 0.2[d]	1.6 ± 0.5[e]
22:4n-6	1.2 ± 0.2[d]	1.0 ± 0.3[d]	0.7 ± 0.2[d]	0.9 ± 0.1[d]	0.4 ± 0.2[e]
22:5n-6	1.0 ± 0.1[d]	0.7 ± 0.1[d]	0.6 ± 0.3[d]	0.2 ± 0.1[e]	trace[d]
Total n-3 FA	trace	1.2 ± 0.1[e]	2.0 ± 0.3[f]	3.0 ± 0.5[f]	5.3 ± 0.5[g]
18:3n-3	trace	0.3 ± 0.0[e]	0.7 ± 0.3[f]	0.9 ± 0.2[f]	2.4 ± 0.7[g]
18:4n-3	trace	0.1 ± 0.0[d,e]	0.1 ± 0.0[d,e]	0.2 ± 0.0[e]	0.3 ± 0.1[e]
20:5n-3	trace	0.2 ± 0.1[d,e]	0.3 ± 0.0[e]	0.4 ± 0.1[e]	0.9 ± 0.1[f]
22:5n-3	trace	0.2 ± 0.1[d,e]	0.3 ± 0.0[e]	0.6 ± 0.0[e]	0.7 ± 0.2[e]
22:6n-3	trace	0.4 ± 0.0[e]	0.6 ± 0.1[e]	0.9 ± 0.1[f]	1.0 ± 0.3[f]
18:2n-6/18:3n-3	ud[e]	18.3 ± 3.7[d]	8.6 ± 3.2[e]	6.6 ± 1.1[f]	3.0 ±0.7[g]
22:5n-6/22:6n-3	ud[e]	1.8 ± 0.4[d]	1.0 ± 0.4[e]	0.2 ± 0.0[f]	0.0[g]

[a,b,c]See footnotes in Table 2. The percentages of the main SFA are: FA ≤ 14:0, 5–6%; 16:0, 22–24%; 18:0, 3%, respectively. Oleic acid (18:1n-9) represents 80% of total MUFA (44% of total fatty acids). The TL contents of colostrum are not significantly different between dietary groups by analysis of variance, and are (mg/g dry matter ± SD, $n = 4$): group A, 641 ± 26; group B, 681 ± 23; group C, 681 ± 45; group D, 689 ± 25; group E, 650 ± 44.
[d]Trace (<0.05% of total FA).
[e]ud, Undetermined.

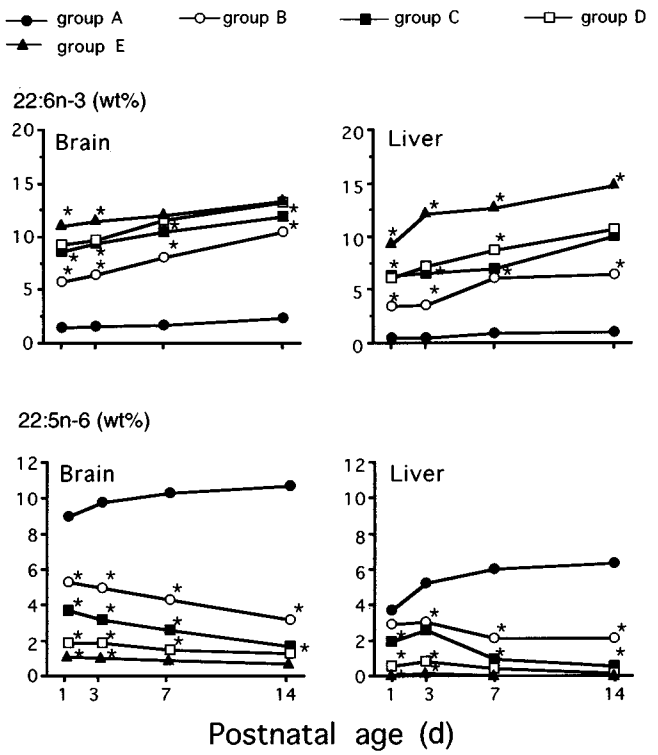

FIG. 2. Effect of the 18:3n-3 content of the maternal diet on the 22:6n-3 and 22:5n-6 contents in brain and liver phospholipids from pups during the first 2 wk of postnatal age. Values are means ± SD (n = 4 in each dietary group). * indicates a significant effect of the 18:3n-3 dietary content ($P < 0.05$) at each time point by analysis of variance, comparisons being as follows: group B vs. group A, group C vs. group B, group D vs. group C, and group E vs. group D. The P values obtained by the two-factor analysis of variance (18:3n-3 dietary content, postnatal age and 18:3n-3 dietary content × postnatal age) on the 22:6n-3 and 22:5n-6 contents in brain and liver are lower than 0.006, except for brain 22:6n-3 where $P = 0.28$ for the diet by postnatal age interaction, and for brain 22:5n-6 where $P = 0.53$ for the postnatal age effect and $P = 0.40$ for the diet by postnatal age interaction.

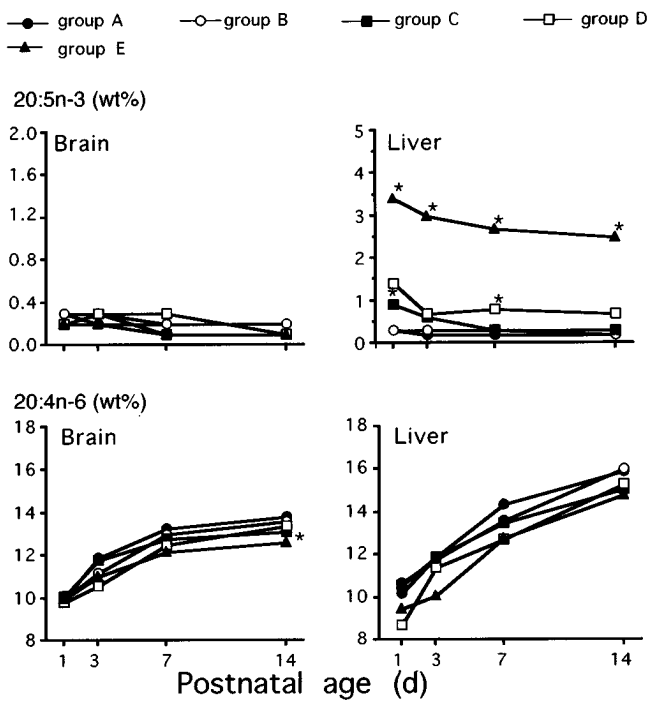

FIG. 3. Effect of the 18:3n-3 content of the maternal diet on the 20:5n-3 and 20:4n-6 contents in brain and liver phospholipids (PL) from pups during the first 2 wk of postnatal age. Values are means ± SD (n = 4 in each dietary group). * indicates a significant effect of the 18:3n-3 dietary content ($P < 0.05$) at each time point by analysis of variance, comparisons being as follows: group B vs. group A, group C vs. group B, group D vs. group C, and group E vs. group D. The P values obtained by the two-factor analysis of variance (18:3n-3 dietary content and postnatal age, 18:3n-3 dietary content × postnatal age) are lower than 0.03, except for brain 20:5n-3 where $P = 0.80$ for the diet by postnatal age interaction, and for 20:4n-6 where $P = 0.37$ for the 18:3n-3 by postnatal age interaction in brain and $P = 0.14$ for the diet effect in liver.

The 22:6n-3 reached the highest (plateau) value on days 7 and 14 postpartum (about 11 and 12.5% of total fatty acids, respectively) only in the brains of pups from dams fed 200 mg/100 g. When the maternal diet supplied at least 200 mg 18:3n-3/100 g, the 22:5n-6 reached the lowest value on days 7 and 14 postpartum, but unlike 22:6n-3, in both the brain and liver (a significant difference was only noted for the brain 22:6n-3 content between group C vs. groups D and E, $P < 0.05$). The percentages of 20:5n-3 and 20:4n-6 in both the tissues were not modified by the 18:3n-3 dietary content except for 20:5n-3 in liver where it rose from 0.2 to 2.5–3.5%.

Milk fatty acid composition during postnatal development. As illustrated in Figure 4, postnatal age-dependent changes in milk fatty acid percentages in all dietary groups were a significant increase in 18:3n-3 and decrease in n-6 and n-3 long-chain derivatives (20:4n-6, 22:5n-6, 20:5n-3, 22:6n-3) ($P < 0.001$). The 18:2n-6 peaked on postpartum day 3 and then returned to its initial level ($P < 0.001$).

No interaction of dietary 18:3n-3 was noted with 18:2n-6 except on day 1 postpartum (Table 3). By contrast, 18:3n-3 regularly increased with the 18:3n-3 dietary content at each time point ($P < 0.001$). In mature milk (Table 4), it represented between 0.5 and 1.2% of total fatty acids when the maternal diet supplied 100 to 400 mg 18:3n-3, and shot up to 3.5% at the highest dietary supply. A significant increase in each n-3 long-chain fatty acid and decrease in each n-6 long-chain fatty acid in interaction with the 18:3n-3 dietary content was noted ($P < 0.001$) (Fig. 4). In mature milk (day 7 postpartum), n-3 represented between 0.2% (group B) and 1.4% (group E) of total fatty acids (Table 4). The 22:6n-3 was the main component except at the highest 18:3n-3 dietary intake where 20:5n-3 represented 50% of long-chain n-3 fatty acids. With varying 18:3n-3 dietary content, postnatal changes in the 22:5n-6 content were inverse to those of 22:6n-3.

As noted in colostrum, the percentage of 18:3n-3 in milk was positively correlated with the dietary 18:3n-3 content ($P < 0.001$) at all times during lactation (data not shown).

FIG. 4. Effect of the 18:3n-3 content of the maternal diet on the n-3 and n-6 fatty acid composition of rat milk total lipids during the first 2 wk of postnatal age. Values are means ± SD ($n = 4$ in each dietary group). * indicates a significant effect of the 18:3n-3 dietary content ($P < 0.05$) at each time by analysis of variance, comparisons being as follows: group B vs. group A, group C vs. group B, group D vs. group C, and group E vs. group D. The P values obtained by the two-factor analysis of variance (18:3n-3 dietary content, postnatal age, 18:3n-3 dietary content × postnatal age) are lower than 0.005 for all fatty acids, except for 18:2n-6 and 20:4n-6 for the 18:3n-3 by age interaction ($P = 0.01$ and $P = 0.03$, respectively). The total lipid contents of rat milk are not significantly modified by the diet and the postnatal age, and are (mg/g dry matter ± SD, $n = 20$): day 1 postpartum, 661 ± 51; day 3 postpartum, 609 ± 32; day 7 postpartum, 618 ± 43; day 14 postpartum, 618 ± 37.

DISCUSSION

Bourre *et al.* (2) concluded that the n-3 fatty acid requirement of the female rat during the entire period of pregnancy–lactation was 200 mg 18:3n-3 per 100 g of maternal food (0.4% of total dietary energy). Indeed they found that the 22:6n-3 content in brain, retina, and synaptosome TL of 21-day-old rats plateaued at this dietary content. The aim of our work was to determine separately the fetal and suckling rat requirements with a larger range of dietary 18:3n-3 content. At birth, we observed that the 22:6n-3 content in pup brain PL increased rapidly up to 200 mg 18:3n-3. Beyond this value, it continued to rise but slowly without reaching a plateau as initially reported in the weaned rat (2). This may reflect a difference of regulation in the DHA synthesis and incorporation into brain PL between the fetal period and the postnatal period. In liver PL, the 22:6n-3 evolution was very similar to that of the brain PL, in agreement with the results of Bourre *et al.* (2). Docosapentaenoic acid (22:5n-6) specifically replaces 22:6n-3 when dietary n-3 fatty acid supply is suboptimal or low (2,11,12). Thus, by analogy with 22:6n-3, the optimal (lowest) value of 22:5n-6 in the brain or liver would be attained when the minimal n-3 fatty acid requirements are covered. As this optimal value of 22:5n-6 was also reached at 800 mg 18:3n-3, we can conclude that the 18:3n-3 requirements of the fetal rat would be at least 800 mg 18:3n-3/100 g of maternal diet. This dietary content appears somewhat excessive because many n-3 long-chain fatty acid intermediates (20:5n-3, 22:5n-3) were incorporated in liver PL and colostrum TL. Moreover, it reduced the level of 20:4n-6 in colostrum TL because of the competition between n-3 and n-6 fatty acids, as suggested by Holmann (18). As 22:6n-3 plateaued at 400 mg 18:3n-3 in colostrum, the accumulation of 20:5n-3 suggests a limited maternal conversion of 20:5n-3 to 22:6n-3 due to an excessive dietary supply of 18:3n-3. A similar high concentration of 20:5n-3 was found in milk from pastoral Chinese women consuming high levels of 18:3n-3 but no seafood (19). Taking into account the 22:6n-3, 20:5n-3, and 22:5n-6 proportions in the pup brain, liver, and maternal milk at birth, we can conclude that the minimal n-3 fatty acid requirement of the female rat during pregnancy is close to 400 mg 18:3n-3/100 g of diet (i.e., 0.9% of total dietary energy, 18:2n-6/18:3n-3 = 3), and that 800 mg has to be considered as an excessive dietary supply.

During the first 2 wk of postnatal life, the 22:6n-3 concentration in brain PL of pups increased regularly and, according to the maternal diet content of 18:3n-3, reached the optimal (plateau) value at 200 mg 18:3n-3/100 g from day 7 postpartum. Conversely, the 22:5n-6 concentration decreased and was the lowest at 200 mg 18:3n-3. These results suggest that the minimal n-3 fatty acid requirement of the suckling rat is 200 mg 18:3n-3/100 g maternal diet (i.e., 0.45% of the dietary energy). The high concentrations of 20:5n-3 in liver PL (>2.5% of total fatty acids) and milk TL (>0.5%) at 800 mg for all the studied periods confirm that this dietary content is excessive. However, the metabolism of n-6 fatty acids was not altered because the accretion of 20:3n-6 and 20:4n-6 in liver and brain remained unchanged, contrary to what happens when lactating mothers consume high levels of 20:5n-3 and 22:6n-3 in the form of fish oil (20). The use of 22:5n-6 in the determination of the n-3 fatty acid requirement is questionable because it seems that this fatty acid is a normal component of brain PL. Taking into account the fivefold lipid increase in the brain during the first 2 wk of postnatal life (240 mg at birth vs. 1.150 mg on day 14 postpartum), the total amount of 22:5n-6 in this organ was increased twofold at the highest 18:3n-3 dietary contents.

TABLE 4
FA Composition of Total Lipids (TL) of Rat Mature Milk (day 7 postpartum) in Relation to the α-Linolenic Content of the Maternal Diet[a]

FA	Dietary group (% total FA)				
	A	B	C	D	E
Total SFA[b]	51.8 ± 3.0	50.1 ± 2.9	49.4 ± 3.2	48.9 ± 2.5	50.3 ± 3.1
Total MUFA[c]	39.9 ± 2.3	41.0 ± 1.8	40.5 ± 1.6	40.3 ± 2.7	36.8 ± 2.2
Total n-6 FA	8.2 ± 0.6	8.0 ± 0.4	8.6 ± 0.6	8.6 ± 0.3	7.6 ± 0.4
18:2n-6	5.8 ± 0.5	6.0 ± 0.2	6.5 ± 0.6	6.3 ± 0.1	6.3 ± 0.2
18:3n-6	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.2	0.2 ± 0.0
20:2n-6	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.1
20:3n-6	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.1
20:4n-6	0.9 ± 0.2	0.7 ± 0.1	1.0 ± 0.2	0.9 ± 0.2	0.6 ± 0.2
22:4n-6	0.4 ± 0.1[d]	0.3 ± 0.2[d]	0.3 ± 0.0[d]	0.3 ± 0.0[d]	0.1± 0.0[e]
22:5n-6	0.2 ± 0.1[d]	0.1 ± 0.0[d]	0.0[e]	0.0[e]	0.0[e]
Total n-3 FA	trace[d]	0.7 ± 0.1[e]	1.2 ± 0.2[f]	2.1 ± 0.2[g]	5.1 ± 0.6[h]
18:3n-3	trace	0.5 ± 0.1[e]	0.7 ± 0.0[e]	1.2 ± 0.1[f]	3.5 ± 0.4[g]
18:4n-3	trace	0.0	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
20:5n-3	trace	0.0[d]	0.1 ± 0.0[d,e]	0.2 ± 0.0[e]	0.7 ± 0.2[f]
22:5n-3	trace	0.0[d]	0.1 ± 0.0[d]	0.3 ± 0.1[e]	0.4 ± 0.2[e]
22:6n-3	trace	0.2 ± 0.1[d,e]	0.2 ± 0.1[e]	0.3 ± 0.1[e]	0.3 ± 0.1[e]
18:2n-6/18:3n-3	ud[e]	12.0 ± 1.0[d]	9.3 ± 1.2[e]	5.3 ± 0.8[f]	1.8 ± 0.4[g]
22:5n-6/22:6n-3	0.0[d]	0.5 ± 0.2[e]	ud	ud	ud

[a,b,c]See footnotes in Table 2. The percentages of the main SFA are: FA ≤ 14:0, 15–18%; 16:0, 30%; 18:0, 2–3%, respectively. Oleic acid (18:1n-9) represents 80% of total MUFA (30–32% of total fatty acids). The TL contents of mature milk are not significantly different between dietary groups by analysis of variance, and are (mg/g dry matter ± SD, $n = 4$): group A, 605 ± 29; group B, 613 ± 24; group C, 627 ± 48; group D, 626 ± 23; group E, 619 ± 36.
[d]Trace (<0.05% of total FA).
[e]ud, Undetermined.

The studies exploring the effects of dietary fatty acids on the membrane composition of the nervous system in the developing rat have investigated the dam's milk composition only on day 14 postpartum (20–23). They give an incomplete view of the quantity of n-3 fatty acids ingested by the suckling pup because 22:6n-3 decreases during the maturation of milk (17,24). The changes in the fatty acid composition of dam's milk were detailed in our study to provide the maximum of data regarding the specific n-3 fatty acid requirements of the suckling rat when the maternal diet contains 200 mg 18:3n-3/100 g. From our data, we estimated the n-3 requirements to be about 0.8–1% of milk total fatty acids for 18:3n-3 and 0.9% (colostrum) to 0.2% (mature milk) for 22:6n-3. On the basis of the weight of milk lipids (mg/g dry matter) found in this study and of the lipid energy concentration of rat milk (25), this corresponds to about 0.5% and 0.1–0.4% of the total dietary energy, respectively. The 18:2n-6/18:3n-3 ratio was close to 9, and systematically 1.7 to 2.4 times higher than in maternal dietary lipids. This higher relative level of 18:2n-6 or lower level of 18:3n-3 in milk has been reported in other studies (20,21). It may result from preferential enrichment in plasma lipids and/or a selective uptake by the mammary gland.

In conclusion, the results of the present study show that pregnant female rats require twice as much dietary 18:3n-3 as lactating rats to satisfy the n-3 requirements of the fetus and suckling pup, respectively. It can also be considered that these increased requirements for dietary 18:3n-3 during pregnancy reflect a specific demand due to their recent n-3 deficient status (i.e., 2 wk before mating). Taking into account the differences in daily dietary intake between these two physiological periods (15 g/d vs. 38 g/d for pregnancy and lactation, respectively) (12), the 18:3n-3 requirements of lactating female rats were 25% higher than those of pregnant female rats (i.e., 75 mg/d vs. 60 mg/d). The n-3 fatty acid contents (18:3n-3 and 22:6n-3) in rat milk which satisfy the requirements of the suckling pup are comparable to those recommended for infant formulas (26,27). The 22:6n-3 content in the rat milk was directly related to the level of 18:3n-3 in the maternal diet, whereas this relation was not observed in human milk (17,24,28). This probably results from dietary habits (presence of 22:6n-3 in the human diet) rather than from species differences. Our data support the use of the suckling rat model to approach the infant fatty acid requirements, but the nutrition of pregnant and lactating rats, the perinatal timing of pup brain growth, and milk fat content and composition are not closely analogous to the human species. In the same way, recent data obtained from the artificially reared rat pup model argued that the rat model is relevant to the design of infant formulas (29), and demonstrated that high dietary intake of 18:3n-3 was unable to achieve adequate neural 22:6n-3 accretion. Future work on the relationship between changes in 22:6n-3 content and function in brain should help to specify the n-3 fatty acid requirements.

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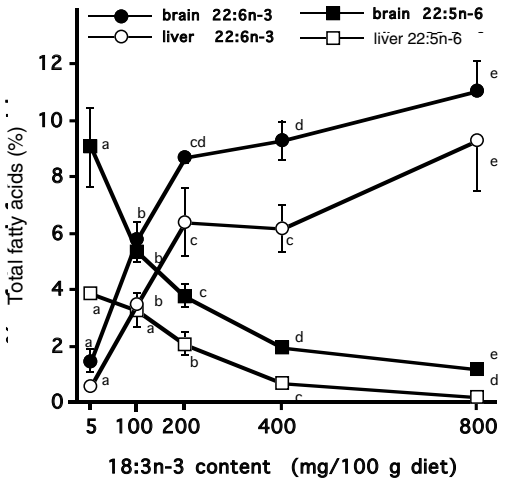
brain 22:6n-3
liver 22:6n-3
brain 22:5n-6
liver 22:5n-6
Total fatty acids (%)
18:3n-3 content (mg/100 g diet)
0
2
4
6
8
10
12
5
100
200
400
800

Lipid Peroxidation During n-3 Fatty Acid and Vitamin E Supplementation in Humans

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ABSTRACT: The purpose of this study was to investigate in healthy humans the effect of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intake, alone or in combination with dL-α-tocopherol acetate (vitamin E) supplements on lipid peroxidation. Eighty men were randomly assigned in a double-blind fashion to take daily for 6 wk either menhaden oil (6.26 g, n-3 fatty acids) or olive oil supplements with either vitamin E (900 IU) or its placebo. Antioxidant vitamins, phospholipid composition, malondialdehyde (MDA), and lipid peroxides were measured in the plasma at baseline and week 6. At the same time, breath alkane output was measured. Plasma α-tocopherol concentration increased in those receiving vitamin E ($P < 0.0001$). In those supplemented with n-3 fatty acids, EPA and DHA increased in plasma phospholipids ($P < 0.0001$) and plasma MDA and lipid peroxides increased ($P < 0.001$ and $P < 0.05$, respectively). Breath alkane output did not change significantly and vitamin E intake did not prevent the increase in lipid peroxidation during menhaden oil supplementation. The results demonstrate that supplementing the diet with n-3 fatty acids resulted in an increase in lipid peroxidation, as measured by plasma MDA release and lipid peroxide products, which was not suppressed by vitamin E supplementation.

Lipids 32, 535–541 (1997).

Fish oils, the most common of which are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown in humans to lower plasma triglycerides (1), to be vasodilatory, to prolong bleeding time (2), and to decrease fibrinogen concentrations (3): all recognized as risk factors for the development of cardiovascular disease. Experimental studies have indicated that the most likely mechanism for the biochemical effects of fish oil relates to the competitive interaction between arachidonic acid (AA) and EPA for incorporation into cellular phospholipids and their further metabolism to eicosanoid products (4).

Fish oils are also highly unsaturated and readily susceptible to peroxidation (5). Peroxidation will give rise to lipid peroxides including malondialdehyde (MDA) and aldehyde breakdown products which have been implicated in the etiology of a number of diseases in humans including cardiovascular disease (6). Vitamin E, because of its antioxidant properties, may reduce the peroxidative damage caused by fish oils and thereby promote its beneficial effects. Fish oil supplements usually contain a small amount of vitamin E as an antioxidant (1 IU), but whether this is sufficient to achieve any beneficial effect is questionable (7).

The purpose of this study was to investigate in humans, in a randomized, double-blind, placebo-controlled fashion, the effect of n-3 fatty acid supplementation, alone or in combination with dL-α-tocopherol acetate (vitamin E), on different lipid peroxidation indices.

MATERIALS AND METHODS

Subjects and protocol. Eighty healthy men (20–60 yr) were recruited, primarily hospital staff and local area workers (social middle class). Informed consent was obtained, and the study protocol was approved by the Toronto Hospital Committee for Research on Human Subjects. Subjects were screened and excluded if they were smokers, had a medical condition (i.e., cancer, heart disease, hypertension, hyperlipidemia, diabetes, obesity, kidney/liver dysfunction, gastrointestinal abnormality, HIV+), had a high alcohol consumption (>30 g/d), or if any vitamin or medications had been taken in the past 4 wk.

The volunteers were also interviewed by a dietitian to ensure that they consumed a diet similar to a typical North American diet (30–40% of energy from fat and providing a polyunsaturated-to-saturated-fatty-acid (PUFA/SFA) ratio of 0.3:1. They were further instructed to avoid all types of fish (i.e., canned, frozen, fresh including shellfish) for the duration of the study and to avoid strenuous exercise.

The subjects returned 2 wk later and were randomized (using random number tables) in a double-blind fashion to supplement their usual diet for 6 wk with either menhaden oil

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Abbreviations: AA, Arachidonic acid; BHT, butylated hydroxytoluene; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; LA, linoleic acid; LDL, low density lipoprotein; MDA, malondialdehyde; OA, oleic acid; PUFA, polyunsaturated fatty acid; Se-GSHPx, selenium-dependent glutathione peroxidase; TBA, thiobarbituric acid; TBHQ, *tert*-butylhydroquinone.

ethyl esters with *tert*-butylhydroquinone (TBHQ: 0.017%) (8 caps/d = EPA 3.062 g, DHA 2.262 g, total n-3: 6.26 g) (National Marine Fisheries Service, Charleston, SC) or olive oil ethyl esters with TBHQ (0.01%) and fish flavoring (8 caps/d = 6.08 g monoenes) with dL-α-tocopheryl acetate (vitamin E; Hoffmann-La Roche Ltd., Basel, Switzerland) (3 caps/d = 900 IU) or its placebo. Subjects were therefore randomized into one of four supplemented groups: menhaden oil + vitamin E; menhaden oil + vitamin E placebo; olive oil + vitamin E; olive oil + vitamin E placebo.

Breath collections and biochemical measurements were performed in the morning, in the fasted state, at baseline (week 0) and at week 6 of supplementation. Blood was collected in tubes containing lithium heparinate for phospholipids; 0.1% EDTA for tocopherols, β-carotene, ascorbic acid, MDA release, and lipid peroxides; additive-free tubes for cholesterol and triglycerides; and trace element-free for selenium-dependent glutathione peroxidase (Se-GSHPx). Samples were centrifuged promptly at 3000 rpm for 10 min. The plasma was removed, aliquoted separately for each assay, and frozen (−70°C) until analysis. For phospholipid analysis, plasma was stored in chloroform containing 0.02% butylated hydroxytoluene (BHT) as an antioxidant, in an oxygen-free environment at −70°C. Plasma for the vitamin C assay was stabilized immediately with 50 g/L metaphosphoric acid (HPO_3) (0.5 mL plasma plus 4.5 mL HPO_3).

Fatty acid analysis. Total lipids were extracted with chloroform/methanol (2:1, vol/vol), containing BHT as an antioxidant (8) and dried under nitrogen gas. The residue then dissolved in 0.1 mL chloroform and applied to silica gel thin-layer plates (Whatman Silica Gel60A K6F, 20 × 20 cm; Interscience Biotechnology, Markham, Ontario, Canada) to separate the phospholipids using a nonpolar solvent system (hexane/diethyl ether/acetic acid; 80:20:1). Phospholipid bands were scraped and transmethylated with boron trifluoride–methanol at 90°C for 30 min. Fatty acid methyl esters were separated on a fused-silica capillary column coated with a 25 μm cyanopropylphenyl film (Durabond 23, 30 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA) in a Hewlett-Packard (HP) 5890A gas–liquid chromatograph (Palo Alto, CA) equipped with a flame-ionization detector (9).

Vitamin analyses. Tocopherols and β-carotene were analyzed using reverse-phase high-performance liquid chromatography (HPLC) and fluorescence spectrophotometry according to the method of Hess *et al.* (10). In this method, the samples are deproteinized by adding 500 μL of ethanol. The samples are then extracted by *n*-hexane and evaporated to dryness at ambient temperature under reduced pressure. The residue is dissolved in 100 μL ethanol/dioxane (1:1) and then 150 μL of acetonitrile is added. The sample extract (100 μL) is then injected into a reverse-phase HPLC. Ascorbic acid was analyzed fluorimetrically by the method of Brubacher Vuilleumier (11). The vitamin concentrations were measured in coded samples by the vitamin research laboratories of F. Hoffmann-La Roche Ltd. (Basel, Switzerland).

Breath analysis. Breath samples were collected and analyzed for ethane and pentane as described previously (12). Briefly, subjects are first required to breathe hydrocarbon-free air for 4 min to wash contaminating hydrocarbons from their lungs. Subsequently, expired air is collected for 2 min and analyzed by gas chromatography (Shimadzu 6-AM GC; Shimadzu Seisgkusho Ltd., Kyoto, Japan) using a Porasil D column (Chromatographic Specialties Inc., Brockville, Canada).

Plasma lipid peroxide analysis. MDA determination was measured by the thiobarbituric acid (TBA) method described by Draper *et al*. (13). Plasma proteins were denatured using 10% trichloroacetic acid solution and adding 0.05% BHT solution prior to digestion on a heating block. After centrifugation, the supernatant was combined with the TBA solution (1:1, vol/vol ratio) and heated. The reaction mixture was then extracted with 1 mL of *n*-butanol. An aliquot of the extract was mixed (2:1:1) with methanol and a mobile phase (15% acetonitrile and 0.6% tetrahydrofuran in 5 mM phosphate buffer) and 20 μL was injected onto a reverse-phase C_{18} μBondpak HPLC column. The standard solution used was 1,1,3,3-tetraethoxy propane (Sigma Chemical Co., St. Louis, MO).

Plasma lipid hydroperoxides were quantitated by calorimetry at 675 nm, measuring the methylene blue formation. Commercial kits (Kamiya Biochemicals Determiner LPO kit, Thousand Oaks, CA) were used.

Se-GSHPx enzyme activity was measured by the coupled enzyme assay (14,15). Changes in absorbance at 340 nm were recorded for 5 min on a spectrophotometer (U 2000 Hitachi Ltd., Tokyo, Japan). Se-GSHPx activity was expressed as μmoles NADPH oxidized $min^{-1} \cdot$ mg $protein^{-1}$ which was measured by the biuret method (16).

Supplement lipid peroxide analysis. A sample of menhaden and olive oil supplements also underwent peroxide analysis at 0 and 18 mon of storage to establish a peroxide value. A microtitrimetric method was used to determine all substances which oxidize potassium oxide in terms of milliequivalents of peroxide per 100 g of sample (17). This analysis was performed by the research laboratories of the National Marine Fisheries Service (Charleston, SC).

Plasma lipid analysis. Serum cholesterol, high density lipoprotein (HDL)-cholesterol, and triglyceride were analyzed using a commercially available kit (Boehringer Mannheim, Montreal, Canada). Low density lipoprotein (LDL)-cholesterol was calculated (cholesterol − (HDL-cholesterol + 0.46 × triglyceride).

Statistical analyses. It was estimated that 20 subjects were required in each group, assuming a dropout rate of 15%, to achieve 80% power using a 5% significance level for detecting at least a 10% difference in lipid peroxidation measured by breath ethane output between groups. All group data are expressed as means ± SEM. The study was a factorial design with the two factors being fish oil and vitamin E. The interaction between fish oil and vitamin E was hypothesized prior to the start of the study such that fish oil would be expected to have an effect on lipid peroxidation, which would not be evident with vitamin E. The comparison of pretreatment and

posttreatment tests was undertaken by an analysis of covariance on the change between baseline (week 0) and week 6 with an adjustment for the week 0 value (18). SAS (19) was used for these analyses.

RESULTS

Of the 80 subjects enrolled, 5 voluntarily withdrew from the trial due to noncompliance and another 3 withdrew because of unpleasant side effects of the therapy. These 3 subjects, all receiving n-3 fatty acid supplements, complained of fishy taste, belching, bloating, heartburn, and nausea. No other significant side effects were reported. Compliance, assessed by plasma α-tocopherol and fatty acids, was very good (Tables 1,2).

Plasma fatty acids and lipids. The fatty acids EPA, DHA, AA, linoleic acid (LA), and oleic acid (OA), expressed as percentage composition of phospholipids, were similar between groups at baseline (Table 2). Phospholipid content of DHA and EPA ($P < 0.001$) increased, and AA and LA composition decreased significantly and to the same extent ($P < 0.025$) in both groups of subjects receiving n-3 fatty acid supplementation. OA increased significantly ($P < 0.025$) and to the same extent in both groups receiving olive oil supplementation.

Serum total triglycerides and cholesterol (HDL-, LDL-cholesterol) showed no significant change (data not shown).

Plasma vitamin concentrations. Plasma α-tocopherol concentrations were comparable between groups at baseline (Table 1). The increase in α-tocopherol in the two vitamin E-supplemented groups ($P < 0.0001$) was significant compared to the two vitamin E placebo groups. Plasma ascorbic acid and β-carotene did not change in any groups during supplementation (Table 1), suggesting that there were no dietary changes during the study.

Lipid peroxidation products in menhaden and olive oil supplements. The peroxide value was 3.27 meq/kg for the menhaden oil supplements and 2.3 meq/kg for the olive oil supplements. An 18-mon storage stability study performed by the manufacturer of the supplements showed no effect of time stored at 5°C on the peroxide value.

Lipid peroxidation measurements in subjects. Breath ethane output was comparable between groups at baseline (Table 3). Supplementation of n-3 fatty acids either with or without vitamin E did not produce any significant changes in breath ethane output. The mean difference in the change of breath ethane output during menhaden oil supplementation between those subjects receiving vitamin E and those not receiving vitamin E supplementation was -1.0 pmol $\cdot$ kg^{-1} $\cdot$ min^{-1} (95% confidence interval, −7.2, 5.2). Breath pentane output did not differ significantly between groups at baseline, and neither n-3 fatty acid nor vitamin E supplementation produced a significant change from initial levels.

Baseline measurements of plasma MDA equivalents, lipid peroxides, and Se-GSHPx were not significantly different between groups (Table 3). In the two groups supplemented with n-3 fatty acids, plasma MDA generation ($P < 0.001$) and lipid peroxides ($P < 0.05$) were significantly increased at 6 wk. Vitamin E intake added to menhaden oil supplements had no effect on MDA or lipid peroxide values. Olive oil supplemented with vitamin E or placebo produced no change in MDA or lipid peroxide levels. Neither n-3 fatty acids nor vitamin E supplementation produced any change in Se-GSHPx activity.

TABLE 1
Plasma Antioxidant Vitamins

	Groups			
	Menhaden oil + vitamin E	Menhaden oil + placebo	Olive oil + vitamin E	Olive oil + placebo
Number	18	17	19	18
Age (yr)	33 ± 2	33 ± 3	32 ± 2	31 ± 2
Initial body mass index	24.7 ± 0.5	25.0 ± 1.1	23.3 ± 0.4	25.4 ± 0.7
α-Tocopherol (μmol/L)				
Week 0	21.3 ± 0.8	21.5 ± 1.0	24.4 ± 0.7	23.6 ± 1.6
Week 6	43.3 ± 2.6	21.4 ± 1.1	52.5 ± 3.4	25.0 ± 1.4
Change (%)	102.7 ± 8.1[a]	−0.3 ± 2.5	115.1 ± 12.9[a]	7.4 ± 2.4
γ-Tocopherol (μmol/L)				
Week 0	3.14 ± 0.24	3.28 ± 0.33	2.83 ± 0.19	2.95 ± 0.28
Week 6	0.82 ± 0.10	2.83 ± 0.20	0.89 ± 0.12	3.12 ± 0.27
Change (%)	−73.4 ± 2.6[a]	−5.6 ± 7.4	−67.3 ± 5.1[a]	7.9 ± 12.0
β-Carotene (μmol/L)				
Week 0	0.43 ± 0.05	0.38 ± 0.04	0.45 ± 0.05	0.38 ± 0.06
Week 6	0.39 ± 0.05	0.42 ± 0.07	0.42 ± 0.04	0.40 ± 0.08
Change (%)	−6.2 ± 5.3	10.1 ± 6.9	−3.2 ± 5.1	5.1 ± 4.9
Ascorbic acid (μmol/L)				
Week 0	26.0 ± 2.7	29.8 ± 2.5	29.8 ± 3.0	25.1 ± 1.9
Week 6	24.9 ± 2.6	27.0 ± 3.0	28.0 ± 3.6	27.9 ± 3.5
Change (%)	−4.0 ± 6.1	−9.1 ± 7.9	−5.4 ± 7.2	11.1 ± 8.3

[a]$P < 0.0001$.

TABLE 2
Plasma Fatty Acid Composition of Phospholipids[a]

	Groups			
	Menhaden oil + vitamin E	Menhaden oil + placebo	Olive oil + vitamin E	Olive oil + placebo
Number	18	17	19	18
EPA (20:5n-3) (composition %)				
Week 0	0.99 ± 0.13	1.07 ± 0.12	1.13 ± 0.09	1.25 ± 0.14
Week 6	8.85 ± 0.56	8.31 ± 0.89	0.99 ± 0.08	1.15 ± 0.11
Change (%)	788.6 ± 83.1[b]	681.3 ± 90.1[b]	–25.2 ± 13.6	–22.9 ± 17.9
DHA (22:6n-3) (composition %)				
Week 0	3.65 ± 0.24	4.15 ± 0.42	3.95 ± 0.24	4.22 ± 0.38
Week 6	8.11 ± 0.26	8.32 ± 0.52	3.32 ± 0.10	3.70 ± 0.23
Change (%)	133.0 ± 12.5[b]	109.7 ± 13.9[b]	–12.6 ± 3.7	–2.1 ± 8.0
AA (20:4n-6) (composition %)				
Week 0	14.23 ± 0.71	13.22 ± 0.70	14.45 ± 0.55	12.66 ± 0.48
Week 6	11.02 ± 0.58	11.13 ± 0.46	13.67 ± 0.49	12.25 ± 0.40
Change (%)	–21.6 ± 3.2[c]	–13.3 ± 4.6[c]	–3.6 ± 4.0	–1.6 ± 4.0
LA (18:2n-6) (composition %)				
Week 0	25.84 ± 0.75	25.84 ± 0.87	23.99 ± 0.65	25.59 ± 1.04
Week 6	18.56 ± 0.65	20.39 ± 0.95	24.15 ± 0.68	24.67 ± 0.85
Change (%)	–27.6 ± 2.7[c]	–20.2 ± 4.2[c]	1.1 ± 2.1	–2.5 ± 3.2
OA (18:1n-9) (composition %)				
Week 0	11.86 ± 0.78	11.13 ± 0.40	11.02 ± 0.41	11.01 ± 0.38
Week 6	10.35 ± 0.58	10.12 ± 0.46	12.39 ± 0.38	12.11 ± 0.37
Change (%)	–11.9 ± 1.6	–8.0 ± 4.8	14.4 ± 4.7[c]	10.7 ± 3.4[c]

[a]Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; LA, linoleic acid; OA, oleic acid; [b]$P < 0.000$; [c]$P < 0.025$.

DISCUSSION

The results of this randomized, double-blind, placebo-controlled study showed that n-3 fatty acid supplementation increased lipid peroxidation based on plasma lipid peroxides and MDA. Menhaden oil intake had no effect on breath alkane output. High doses of vitamin E did not provide adequate antioxidant protection as the lipid peroxidation products remained elevated.

The choice of dosage (6 g of n-3 fatty acids daily) used in this trial was based on studies investigating the effect of fish oil suppplementation on cardiovascular risk factors and lipid peroxidation parameters (20–23). A dose-response curve study (20) showed that in healthy humans 6 g of n-3 fatty acids had a beneficial effect on plasma phospholipid composition and triglyceride levels. However, 6 g had no effect on functional parameters such as blood pressure, bleeding time, erythrocyte deformability, or neutrophil function. For this reason, we did not measure functional parameters in addition to lipid peroxidation. Since our study's main endpoint was lipid peroxidation, 6 g a day of n-3 fatty acids was chosen on the basis of previous reports showing an increase in lipid peroxidation (22,23).

The amount of vitamin E supplementation used was also based on previous literature. Despite 400 IU of vitamin E, some investigators have found that 2.0 g EPA and 1.3 g DHA daily can increase plasma lipid peroxides and MDA (24,25). Our own studies in smokers, an oxidatively stressed population, demonstrated that vitamin E, 800 IU per day, had a significant effect in reducing lipid peroxidation (26). Thus a dose in similar range should have had an effect on lipid peroxidation during n-3 fatty acids supplementation. Data from the literature also indicate that for each gram of linoleic acid in the diet, a minimum additional requirement of 0.5 mg of α-tocopherol is desirable (27) to prevent lipid peroxidation. Based on Muggli's equation (27), 2.24 and 2.68 mg of supplementary dL-α tocopherol is needed for each gram of dietary EPA and DHA, respectively. Thus, the amount of α-tocopherol supplementation in our study should have been between 10 and 15 IU per day, a far lower dose than what we used.

The increase in plasma MDA and lipid peroxide levels observed during fish oil supplementation suggests that the antioxidant level, whether in the plasma or in the capsules, was not sufficient to protect against free radical generation. Ingestion of lipid peroxide products from the capsules would explain the persistent elevation in these lipid peroxidation indices despite vitamin E supplementation. This could also explain the lack of induction of Se-GSHPx enzyme activity since lipid peroxidation would have occurred *ex vivo*. However, in the menhaden and olive oil supplements, the peroxide value measuring the oxidation was within acceptable limits and was comparable between the two. To prevent oxidative deterioration of n-3 fatty acids, the American Institute of Nutrition recommends the addition of a synthetic antioxidant at a level of 0.02% (by weight of oil) to experimental diet containing polyunsaturated fatty acids (PUFA) (28) which was the amount of TBHQ added to our supplements. How-

TABLE 3
Lipid Peroxidation Indices[a]

	Groups			
	Menhaden oil + vitamin E	Menhaden oil + placebo	Olive oil + vitamin E	Olive oil + placebo
Number	18	17	19	18
Breath ethane output (pmol · kg^{-1} · min^{-1})				
Week 0	12.2 ± 1.4	11.0 ± 1.0	11.3 ± 1.0	11.2 ± 1.1
Week 6	10.3 ± 1.4	11.7 ± 1.5	12.5 ± 1.7	12.8 ± 1.6
Change (%)	−15.5 ± 13.8	5.3 ± 12.1	7.1 ± 10.9	15.1 ± 19.3
Breath pentane output (pmol · kg^{-1} · min^{-1})				
Week 0	6.3 ± 1.2	5.3 ± 0.7	5.1 ± 0.6	7.5 ± 1.6
Week 6	5.4 ± 0.8	5.8 ± 1.0	4.6 ± 0.5	5.5 ± 1.1
Change (%)	−14.3 ± 12.2	9.4 ± 14.6	−9.3 ± 10.8	−16.7 ± 19.8
MDA equivalent (nmol/mL)				
Week 0	1.87 ± 0.15	1.91 ± 0.13	1.91 ± 0.12	1.81 ± 0.16
Week 6	3.13 ± 0.38	3.06 ± 0.32	1.92 ± 0.13	1.59 ± 0.13
Change (%)	67.3 ± 15.3[b]	60.7 ± 15.9[b]	2.6 ± 2.2	−5.8 ± 5.2
Lipid peroxides (nmol/mL)				
Week 0	316.2 ± 17.4	274.0 ± 25.4	367.9 ± 19.2	346.6 ± 20.9
Week 6	407.0 ± 24.8	324.1 ± 17.5	359.8 ± 22.7	348.0 ± 24.2
Change (%)	34.6 ± 10.9[c]	21.5 ± 8.8[c]	2.6 ± 2.1	0.4 ± 1.0
Se–GSHPx (μmol NADPH · min^{-1} · mg $protein^{-1}$)				
Week 0	4.5 ± 0.2	4.7 ± 0.3	4.6 ± 0.3	4.4 ± 0.2
Week 6	5.2 ± 0.2	4.1 ± 0.2	4.5 ± 0.3	4.6 ± 0.3
Change (%)	15.1 ± 5.1	−14.6 ± 7.1	−1.9 ± 4.1	2.1 ± 7.8

[a]Abbreviations: Se-GSHPx, selenium-dependent glutathione peroxidase; MDA, malondialdehyde; [b]$P < 0.001$; [c]$P < 0.05$.

ever, a recent study by Gonzalez *et al*. (29) suggested that this may not be sufficient to totally prevent oxidative deterioration, at least in animal diets high in fish oil. In that study, the addition of even 100 times this level (2% TBHQ), although decreasing the level of oxidation products, failed to totally prevent lipid peroxidation. These experiments were performed with purified animal diets and may not be applicable to the supplements used in our study. However, although the peroxide values measured in our supplements did not increase when tested, storage condition may not have been optimal once the supplements were given to the subjects. This could have induced lipid peroxidation within the supplements. Whether ingestion of lipid peroxidation products could have significantly increased plasma levels in humans is, however, not clear. Animal studies (29) have shown a significant increase in thiobarbituric acid reactive substances (TBARS) concentrations of different organs and whole body when mice were fed fish oil diet without antioxidants. The addition of high levels of antioxidants to the diet actually reduced these levels.

The increase in plasma MDA and lipid peroxides due to n-3 fatty acid supplementation with or without vitamin E intake was reported in small studies. Some reported that high intakes of fish oil concentrate led to increased TBARS irrespective of the vitamin E intake (24) while others reported a decrease in TBARS after fish oil supplementation with additional vitamin E (25). Both studies compared end point values to baseline and were not randomized. Another study (7) investigated the effect of long-term fish oil supplementation in women. There was a significant increase in plasma lipid peroxides while plasma vitamin E concentrations did not change. Similar results for plasma vitamin E levels were found in males (30). A double-blind, crossover study (31), conducted in 12 healthy volunteers, showed a decrease in plasma vitamin E and an increase in MDA with fish oil supplemented with 0.3 IU/g of vitamin E while levels of both plasma vitamin E and MDA remained normal with 1.5 IU/g. Peroxide values of both oils remain similar during storage, suggesting that lipid peroxidation occurred *in vivo*. Other studies in animals showed an increase in lipid peroxidation along with a reduction in antioxidant defense systems (32–35). Possible effects from lipid peroxidation products within the food cannot be completely ruled out (29,36).

No change in breath ethane output was detected in this study. Breath ethane output has received less attention in human studies as a measure of lipid peroxidation, likely because pentane represents the majority of PUFA (n-6) in the body (37). However, breath ethane output is a well-recognized index of lipid peroxidation (38–40) and has been reported to be elevated in smokers (41) and children with liver disease (42). But to our knowledge, other then the present report, there are no human studies published on dietary intake of PUFA and breath alkane output. On the other hand, in animals, increased production of ethane was demonstrated with high intake of n-3 fatty acids from cod liver oil (43). In our study with humans, this effect could not be demonstrated with menhaden oil supplementation. More extreme changes in n-3 vs. n-6 fatty acid intake may have been necessary in order to detect a change in ethane output. We attempted to control for

other factors known to influence breath alkane output such as the PUFA/saturated fatty acid ratio of the diet (39), exercise levels (44), and alcohol consumption (45,46) in order to reduce the inter- and intrasubject variability. However, unlike animal studies (43) done in a laboratory environment, this study was not performed in a clinical investigation unit, and thus strict control of these variables was not possible.

Vitamin C and β-carotene were measured in the plasma to ensure that concentrations remained stable throughout the study. Since these vitamins have an antioxidant effect, changes occurring because of sudden increase in intake could have influenced our results.

In conclusion, this study has shown that supplementing the diet with 6 g of n-3 fatty acids daily resulted in increased lipid peroxidation as measured by plasma MDA equivalents and lipid peroxides. However, breath ethane output did not increase and, in humans, may not be a method sensitive enough to measure lipid peroxidation due to changes in PUFA intake. Vitamin E supplementation (900 IU/d) did not protect against lipid peroxidation during n-3 fatty acid supplementation. The possibility of increased lipid peroxide products within the capsules, while stored by the subjects, could not be excluded.

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The Pharmacokinetics of Water-in-Oil-in-Water-Type Multiple Emulsion of a New Tacrolimus Formulation

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ABSTRACT: We developed a water-in-oil-in-water (W/O/W)-type multiple emulsion of a new tacrolimus formulation. A potential approach to avoid the complications of systemic immunosuppression and simultaneously enhance immunosuppressive efficacy is to deliver immunosuppressive agents locally to the site of the target organs. The W/O/W emulsion is dispersed oil drops containing smaller water droplets that allow the delivery of drugs preferentially to the reticuloendothelial system (RES). Since the liver and the spleen are primary components of the RES, and the brain and the kidney have a poor RES, we hypothesized that a W/O/W emulsion of tacrolimus would possess the pharmacokinetic benefits of local immunosuppression. We evaluated this hypothesis in a rat model. The tacrolimus levels of whole blood, the liver, spleen, brain, and kidney in rats given intravenous emulsions of tacrolimus (W/O/W group) were compared with a group administered tacrolimus alone (T group). There were no significant differences between the pharmacokinetic parameters of W/O/W group and T group based on whole blood data. However, the W/O/W group had significantly decreased tacrolimus levels in the brain and kidney, and significantly increased levels in the liver and spleen compared with the T group. These data suggest that the W/O/W emulsion is applicable as an intravenous drug carrier for local immunosuppression.
Lipids 32, 543–548 (1997).

Tacrolimus, a macrolide with a mechanism of action similar to that of cyclosporine (1), has been widely studied in clinical trials and was approved for use in patients undergoing liver transplantation in April 1994. Tacrolimus is a very powerful immunosuppressive drug which has increased graft and patient survival in organ transplantation (2). However, primarily nephrotoxic and neurotoxic effects are three times more frequent in tacrolimus-treated patients than in cyclosporin-treated patients, restricting the permissible dosage of tacrolimus (3). A potential approach to reduce the adverse effects of systemic immunosuppression and simultaneously enhance the immunosuppressive efficacy is to deliver the immunosuppressants locally to the site of the target organs.

We developed a new formulation of tacrolimus by taking advantage of its suspension in a water-in-oil-in-water (W/O/W)-type multiple emulsion. This is a system of dispersed oil drops containing even smaller water droplets in which the tacrolimus is dissolved. Investigators in the pharmaceutical field have been particularly interested in the development of slow-release drug delivery systems (4,5) and in selectively transferring drugs into the lymphatic system using W/O/W emulsions (6). Although the W/O/W-type multiple emulsion was first reported by Herbert (7) in 1965, W/O/W emulsions have never been used intravenously because of the toxicity of oils and surfactants. Our W/O/W emulsion was prepared by a method different from that in Herbert's original report (7).

Local immunosuppression methods have been shown to achieve the goals of preventing rejection in rodent and canine models with reduced systemic drug exposure and toxicity (8). Drug targeting systems such as drug-impregnated polymer rods, (9) controlled-release matrices, (10) and liposomes (11) have been used to direct immunosuppressive agents to the transplanted target organs in rodent and canine models. Liposomal incorporation is a useful method for increasing the immunosuppressive efficacy of immunosuppressants in liver transplantation models (11,12).

We suspected that a W/O/W-type multiple emulsion of tacrolimus was unlikely to be distributed in the kidney and brain, with their poor reticuloendothelial system (RES), and was more likely to be distributed in the liver and spleen, with their rich RES. To reduce the adverse effects of tacrolimus without diminishing its immunosuppressive effects, we developed a W/O/W-type multiple emulsion which incorporated tacrolimus.

In the present study, a W/O/W-type multiple emulsion based in soybean oil was used as a model for the delivery of tacrolimus. The pharmacokinetic benefits and potential usefulness of the emulsion as a carrier were evaluated.

MATERIALS AND METHODS

Materials. Tacrolimus and soybean oil were purchased from Fujisawa Pharmaceutical Company Ltd. (Osaka, Japan) and

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Abbreviations: AUC, area under the concentration time curve; β, apparent elimination rate constant; β-phase apparent volume of the distribution; CL_{tot}, total clearance; C_p, whole blood tacrolimus concentration; GE, glycerol fatty acid ester; RES, reticuloendothelial system; TEM, transmission electron microscope; $t_{1/2\beta}$, biological half-life of the elimination phase; V_c, apparent volume of the central compartment; $V_{d(\beta)}$, W/O/W, water-in-oil-in-water.

Wako Pure Chemical Ltd. (Osaka, Japan), respectively. Glycerol fatty acid ester (GE; Sunsoft No.818 NSM, Taiyo Chemical Co., Yokkaichi, Japan), lecithin from egg (Wako), and Pluronic F68 (Green Cross Pharmaceutical Co., Osaka, Japan) were used as surface agents. GE and lecithin are used as food additives, and Pluronic F68 has been approved as the stabilizer of red blood cell membrane.

Preparation of emulsion. This study used tacrolimus solution (10 mg/1 mL) containing an appropriate amount of ethanol and 400 mg castor oil derivative (HCO-60) for intravenous administration of tacrolimus. Tacrolimus solution (10 mg/1 mL) was diluted with 9 mL isotonic sodium chloride solution. To prepare the water-in-oil emulsion, 10 mg/10 mL tacrolimus solution was simply combined with a mixture of soybean oil, lecithin, and GE (tacrolimus solution/soybean oil/lecithin/GE, 10:18:1:1, mL/mL/g/mL) and stirred for about 15 min in an ice bath using an ultrasonic homogenizer (V-Level 200 μA; Nippon Seiki Co., Tokyo, Japan). The water-in-oil emulsion (30 mL) was dispersed in 70 mL of isotonic sodium chloride solution containing 3.0% (wt/vol) Pluronic F68, and then stirred with an ultrasonic homogenizer (V-Level 200 μA) for 10 min. W/O/W emulsions were microscopically checked by microphotography and transmission electron microscope (TEM). The diameter of the W/O/W emulsion was measured by a Shimazu Laser Diffraction Particle Size Analyzer, SALD-2001 (Kyoto, Japan). To determine the entrapping efficiency of tacrolimus in the internal aqueous phase of W/O/W emulsion, 3.0 mL of 6.0% (wt/vol) hydroxy ethylstarch (Kyourin Pharmaceutical Co., Tokyo, Japan) was added to 1.0 mL of the aqueous phase of the W/O/W emulsion which was separated by centrifugation (at 5°C, 4500 × *g*, 10 min). After centrifugation (at 5°C, 4500 × *g*, 10 min), the tacrolimus concentration in the separated external water was assayed by the enzyme immunoassay of Tamura *et al.* (13). The entrapping efficiency was calculated by the equation:

$$\text{entrapping efficiency (\%)} = 100 \cdot (A - 4 \cdot C_{ext} \cdot V_{ext})/A \qquad [1]$$

where A represents the values for total amount of tacrolimus in the W/O/W emulsion, C_{ext} represents the values for tacrolimus concentration in the separated external water of W/O/W emulsion, and V_{ext} represents the volume of the external aqueous phase of W/O/W emulsion.

Animals. All animals were housed, fed, and handled in accordance with the "Guidelines for Animal Experimentation of Hamamatsu University School of Medicine" (Shizuoka, Japan), with approval of the institution review board for animal experiments. Male DA/Slc rats weighing 180–220 g (SLC, Shizuoka, Japan) were used. The experiments described in this report were conducted according to the "Guide for the Care and Use of Laboratory Animals," and it is required that research adhere to the Declaration of Helsinki.

Experimental design. Animals were separated into two experimental groups. The first (W/O/W group) consisted of rats given the W/O/W emulsion of tacrolimus. The second group contained rats given 1 mg/10 mL tacrolimus solution which was diluted with isotonic sodium chloride solution (T group). Tacrolimus was administered intravenously into a cutaneous pedis vein at a dose of 1.0 mg/kg (W/O/W group; $n = 40$, T group; $n = 40$) under ethyl ether anesthesia. The test solution contained 0.1 mg of tacrolimus in 1 mL in each group. Venous blood samples and tissue samples were obtained predose ($n = 5$) and then 5 min ($n = 5$), 10 min ($n = 5$), 15 min ($n = 5$), 30 min ($n = 5$), 1 h ($n = 5$), 2 h ($n = 5$), and 6 h ($n = 5$) after drug administration. After laparotomy, blood samples were taken from the inferior vena cava and were collected in EDTA-containing tubes. Subsequently, brain, liver, spleen, kidney, and lung samples were excised. These unrinsed samples were immediately frozen at −20°C. Lungs were removed 6 h after drug administration and fixed in 10% formaldehyde solution. Then the lungs were prepared with Sudan-III stain for light microscopy.

Measurement of tacrolimus levels. Concentrations of tacrolimus in whole blood and tissue samples were assayed by enzyme immunoassay method using antitacrolimus monoclonal antibody as previously described (13). Rat whole blood standard and tissue samples standards in the range of 0.5–50 ng/mL were prepared using blank rat whole blood and homogenates of tissue samples. The standard curves were shown by four-parameter-fitting equation:

$$Y = (A - D)/\{1 + (X/C)^B\} + D \qquad [2]$$

where Y represents the values for optimal density, X represents the values for concentration of tacrolimus, and A, B, C, and D represent the values for parameter. The correlation coefficients of whole blood and tissue samples were more than 0.998 in both the free and emulsion forms of tacrolimus. For the determination of tissue levels of tacrolimus, tissue samples were homogenized in 10 vol of isotonic sodium chloride solution and analyzed. Tissue tacrolimus levels were calculated as the amount of tacrolimus in 1 g of tissue sample. We performed the control experiment that homogenized both brain and liver in the same mixture to account for intrinsic differences between the tissues' lipophilicity. Brain extractions were compared with liver extractions containing tacrolimus in the same concentration range. As shown in Figure 1, there was no significant effect of tissues' lipophilicity on the enzyme immunoassay for tacrolimus. Whole blood tacrolimus concentration (C_p) profiles were described by polyexponential equations:

$$C_p = \sum C_i e^{\lambda_i t} \qquad [3]$$

where $i = 2$ for T group and W/O/W group in whole blood. C_i and λ_i denote intercept coefficients and slopes. Data were subjected to nonlinear regression analysis. The area under the whole blood concentration–time curve (AUC) was calculated from the slopes and coefficients (AUC = $\sum C_i/\lambda_i$). The total

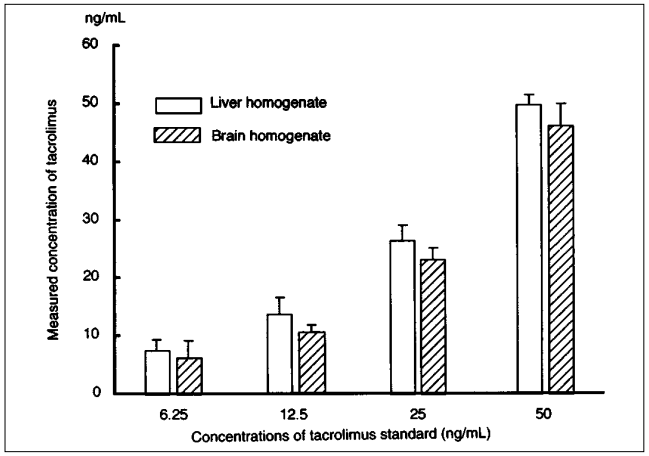

FIG. 1. Comparison of tacrolimus concentrations of standards with homogenized brain ($n = 3$) and liver ($n = 3$) in the same mixture. Values represent mean ± SD. There is no significant difference between tacrolimus concentrations extracted from liver and brain.

clearance (CL_{tot}) was calculated from Dose/AUC. The apparent volume of the central compartment (V_c) was obtained from Dose/$\sum C_i$. The β-phase apparent volume of distribution ($V_{d(\beta)}$) was calculated from CL/apparent elimination rate constant (β). The half life of the elimination phase ($t_{1/2\beta}$) was calculated from 0.693/β.

Statistics. Tacrolimus levels are presented as means ± standard deviation, and compared between the groups using the unpaired Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

The morphological findings of W/O/W emulsion droplets were demonstrated by TEM (Fig. 2). Each droplet contained many smaller water droplets. The mean diameter of the W/O/W emulsion droplets was 3.21 ± 0.10 μm. The entrapping efficiency of tacrolimus was 84.0 ± 4.0%, and did not change when the emulsion was stored at 5°C for 5 d.

Concentration of tacrolimus in blood. Mean whole blood concentration profiles of drug after intravenous injection of W/O/W group and T group at a dose of 1 mg/kg are shown in Figure 3 and Table 1. Whole blood profiles were described using a biexponential equation for both W/O/W group and T group in Figure 3. Whole blood concentrations of tacrolimus were significantly higher (5 min, 15 min, and 30 min: $P < 0.01$, 10 min: $P < 0.05$) in the W/O/W group than in the T group (Table 1). Table 2 lists the pharmacokinetic parameters based on whole blood concentration. There were no significant differences between the pharmacokinetic parameters of W/O/W group and T group.

Tacrolimus levels of organs. As shown in Table 3, the tacrolimus levels in the liver at 15 min were significantly higher ($P < 0.01$) in the W/O/W group than in the T group. But there were no significant differences between the liver concentration of W/O/W group and T group at 1 and 6 h, showing a marked decrease of liver concentration at 1 h in W/O/W group. The tacrolimus levels of the spleen in the W/O/W group were also significantly higher (15 min and 6 h: $P < 0.05$, 1 h: $P < 0.01$) than in the T group. In contrast, the tacrolimus levels in the brain were quite low compared with the levels in other organs at 15 min and 1 h after injection.

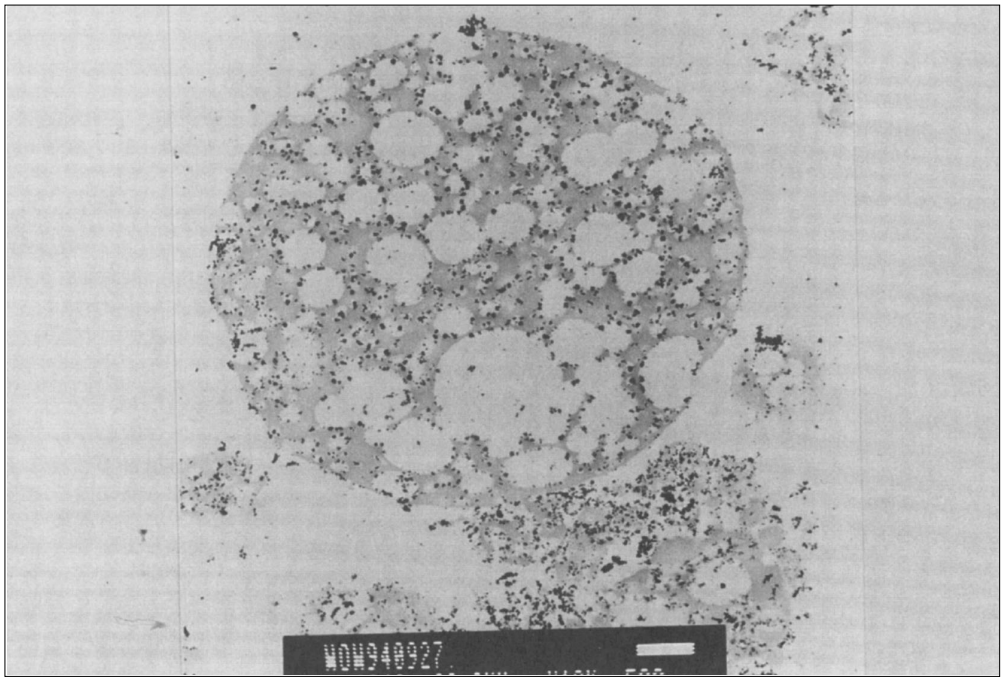

FIG. 2. Electron microscopic appearance of W/O/W-type multiple emulsion, which contains many smaller water droplets, scale bar = 500 nm.

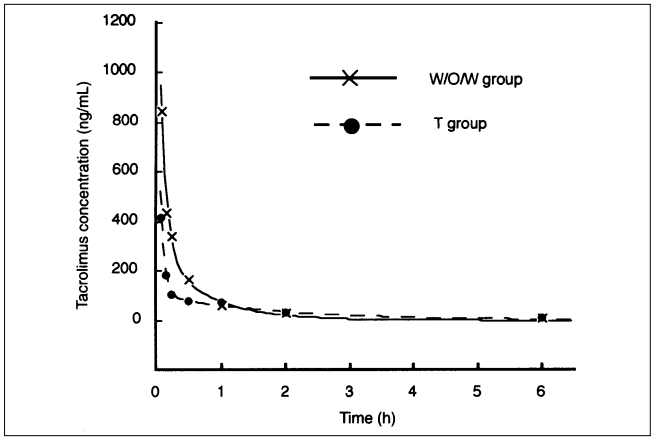

FIG. 3. Whole blood tacrolimus concentration profiles of W/O/W group and T group following single intravenous doses of 1.0 mg/kg to rats. (X), water-in-oil-in-water group (n = 5); (●), tacrolimus solution group (n = 5). Each point represents the mean of five rats. Solid and dotted lines represent curve-fitting of data to polyexponential equations. These profiles were described using a biexponential equation for both groups.

The brain concentrations at 1 h and 6 h were also significantly lower (1 h: $P < 0.05$; 6 h: $P < 0.01$) in the W/O/W group than in the T group. The tacrolimus levels in the kidney in the W/O/W group were significantly lower (15 min: $P < 0.05$, 1 h and 6 h: $P < 0.01$) than those in the T group. Also there were no significant differences between the lung concentration of tacrolimus in the W/O/W group and in the T group.

Histological findings of lung. Gross inspection at autopsy showed no change of lungs macroscopically, but the histological sections of lung in some samples microscopically demonstrated mild deposits of fat in the pulmonary blood vessels (Fig. 4).

DISCUSSION

This is the first study to document the pharmacokinetic benefits of an intravenously administered W/O/W emulsion incorporating tacrolimus. A W/O/W-type multiple emulsion was used as a carrier of tacrolimus *via* an intravenous route for the following reasons: W/O/W emulsions enter the lymphatic system easily (6); tacrolimus can be enclosed both easily and efficiently with multiple emulsion. To prepare the W/O/W emulsion, we used castor oil derivative (HCO-60) which may cause allergic symptoms (14,15). The feasibility of W/O/W emulsion as an alternative to the tacrolimus intravenous dosage form without HCO-60 should be assessed. The mean particle size of 3.21 μm is not small enough to avoid trapping in the lung. But the lung concentrations of tacrolimus were not significantly different between the W/O/W group and T group. Fat accumulation in the lung may not result in major changes in lung distribution. Further refinements in the formulation of this W/O/W emulsion would likely result in smaller particles and decreased fat accumulations in the lung. Toxicity was not investigated in this study because single intravenous doses of tacrolimus are not likely to evoke toxicity.

The pharmacokinetic parameters were calculated by whole blood concentrations. However, no significant differences be-

TABLE 1
Tacrolimus Concentration (ng/mL) in Whole Blood

	Time (min)						
Group	5	10	15	30	60	120	360
W/O/W[a]	847 ± 238[b]	435 ± 177[c]	340 ± 67[b]	163 ± 20[b]	61 ± 22	27 ± 7	7 ± 1
T[a]	413 ± 130	181 ± 40	104 ± 18	77 ± 20	73 ± 35	28 ± 9	6 ± 3

[a]Tacrolimus (1mg/kg) was administered as each formula; W/O/W, water-in-oil- in-water; T, tacrolimus solution. Values represent mean ± SD of five animals. Significantly different from T group.
[b]$P < 0.01$.
[c]$P < 0.05$.

TABLE 2
Pharmacokinetic Parameters[a] Based on Whole Blood Concentrations of Tacrolimus After Single Intravenous Dose

Group	AUC[b] 0–6 h (ng h/mL)	CL_{tot}[c] (L/h/kg)	V_c[d] (L/kg)	$V_{d(\beta)}$[e] (L/kg)	$t_{1/2\beta}$[f] (h)
W/O/W[g]	369.2 ± 86.9	2.84 ± 0.69	0.90 ± 0.34	7.52 ± 1.61	1.85 ± 0.12
T[g]	265.8 ± 44.9	3.84 ± 0.65	0.94 ± 0.23	9.18 ± 1.71	1.73 ± 0.28

[a]Values represent mean ± SD of five animals. There were no significant differences between the pharmacokinetic parameters of W/O/W group and T group.
[b]AUC 0–6 h, area under the concentration–time curve.
[c]CL_{tot}, total clearance.
[d]V_c, apparent volume of the central compartment.
[e]$V_{d(\beta)}$, β-phase apparent volume of distribution.
[f]$t_{1/2\beta}$, half life of the elimination phase.
[g]W/O/W, water-in-oil-in-water; T, tacrolimus solution. Tacrolimus (1mg/kg) was administered as each formula.

TABLE 3
Changes in Time of Tacrolimus Levels (ng/1 g tissue) in Organs[a]

Time	Group	Tacrolimus level (ng/1 g tissue)				
		Liver	Spleen	Brain	Kidney	Lung
15 min	W/O/W[b]	1950 ± 354[c]	1670 ± 344[d]	60 ± 24	1450 ± 212[d]	1325 ± 21
	T[b]	507 ± 76	1101 ± 57	44 ± 5	1800 ± 100	1394 ± 269
1 h	W/O/W[b]	197 ± 15	700 ± 95[c]	27 ± 1[d]	490 ± 17[c]	1007 ± 19
	T[b]	193 ± 12	500 ± 78	48 ± 6	660 ± 69	969 ± 181
6 h	W/O/W[b]	35 ± 5	371 ± 51[d]	29 ± 4[c]	190 ± 10[c]	406 ± 170
	T[b]	48 ± 3	308 ± 40	53 ± 2	250 ± 0	434 ± 151

[a]Values represent mean ± SD of five animals.
[b]Tacrolimus (1 mg/kg) was administered as each formula. W/O/W, water-in-oil-in-water; T, tacrolimus solution. The value is significantly different from T group.
[c]$P < 0.01$.
[d]$P < 0.05$.

tween W/O/W group and T group in the pharmacokinetic parameters were found. Whole blood concentration profile was reported to be best fitted by triexponential equation for conventional intravenous dosage form of tacrolimus (16). Differences in pharmacokinetics at steady-state (γ phase) should be confirmed by whole blood concentrations in the W/O/W group and T group. The present pharmacokinetic study of the W/O/W group demonstrated significantly increased tacrolimus levels in the liver and spleen, and significantly decreased tacrolimus levels in the kidney and brain, relative to the T group. This indicates that the main site of W/O/W emulsion uptake after intravenous injection was the RES. The brain and kidney contain little in the way of RES, which may be the reason that the administration of W/O/W emulsion reduced the tacrolimus levels in those organs. A study of liposomal immunosuppressants has previously described the same distributions of immunosuppressants to these organs (17). Because the liver is the site of the allograft in liver transplantation and the spleen is a major lymphoid tissue, a W/O/W emulsion of tacrolimus may increase the immunosuppressive efficacy in liver transplantation. However, enhancement of the immunosuppressive effect by a W/O/W emulsion of tacrolimus will have to be confirmed in studies of experimental transplantation.

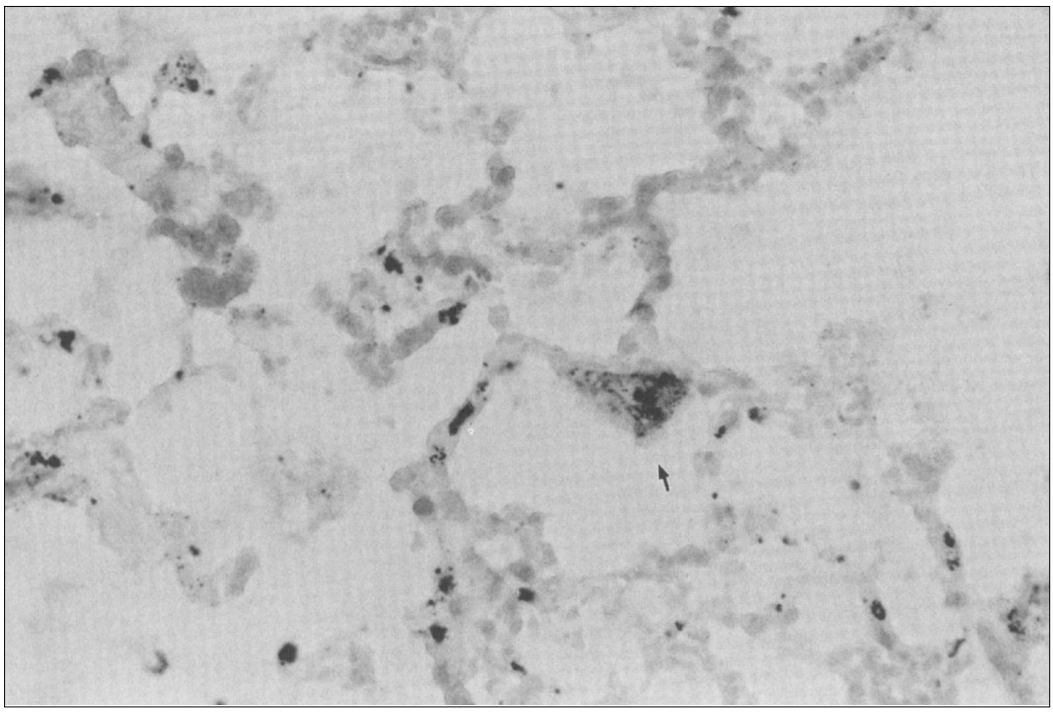

FIG. 4. A branch of the terminal bronchiole 6 h after administration of tacrolimus in the W/O/W group. The arrow indicates the site of fat deposit in pulmonary blood vessels (Sudan-III stain, ×100).

From the viewpoint of adverse effects, decreased tacrolimus levels in the kidney and brain following a W/O/W emulsion of tacrolimus may alleviate the nephrotoxicity and neurotoxicity. Our results suggest that the tacrolimus concentration in the brain was quite different from that in other organs because the brain is protected by the blood brain barrier. Other investigators working with tacrolimus delivered in liposomes have demonstrated decreased tacrolimus levels in the brain (17), but a recent study revealed that the brain had an extraordinarily high density of tacrolimus-binding protein, to which tacrolimus binds with high affinity (18). We need to explore whether reduced tacrolimus levels in brain tissue as a result of delivering it *via* W/O/W emulsions will result in contribution to decreased neurotoxicity. McCarley *et al.* (19) demonstrated that the nephrotoxicity of tacrolimus was dose-dependent, and that measures to decrease the quantity of drug required would reduce drug-induced renal damage. A previous study of local immunosuppression found that CsA liposomes had potential for use as an immunosuppressive agent with decreased nephrotoxicity relative to the commercially available form of intravenous CsA in an ischemic kidney model in the rat (11). The reduction of tacrolimus levels in the kidney by a W/O/W emulsion would be expected to reduce the nephrotoxicity of tacrolimus.

The use of immunosuppressive agents incorporated in a W/O/W-type multiple emulsion is a potentially useful method for the local delivery of the agents to target organs. By taking advantage of the concept of local immunosuppression, it may be possible to increase the immunosuppressive efficacy and decrease the adverse effects of systemically administered immunosuppressants. The optimal emulsion size, the likely increased therapeutic index of tacrolimus in experimental liver transplantation, and the alleviation of neurotoxicity and nephrotoxicity should be evaluated by further investigations in animal models.

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Structure Analysis of an Allene-Containing Estolide Tetraester Triglyceride in the Seed Oil of *Sebastiana commersoniana* (Euphorbiaceae)[1]

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ABSTRACT: The seed oil of *Sebastiana commersoniana* (Euphorbiaceae) was separated into a triglyceride and an estolide fraction by preparative thin-layer chromatography. The triglyceride band was characterized by spectroscopic methods, and its fatty acids have been analyzed by gas chromatography (GC) and GC–mass spectrometry (MS) as their methyl esters. Linolenic acid was the main fatty acid (65%). The estolide band was examined by a combination of spectroscopic and chromatographic methods (ultraviolet, infrared, nuclear magnetic resonance, fast atom bombardment–MS, GC–MS of the fatty acids before and after silylation) and was identified as a tetraglyceride, where one α-carbon of the glyceride backbone was esterfied with 8-hydroxy-5,6-octadienoic acid, which itself was esterfied with *trans*-2,*cis*-4-decadienoic acid. The remaining positions of the glyceride backbone were occupied by common fatty acids.

Lipids 32, 549–557 (1997).

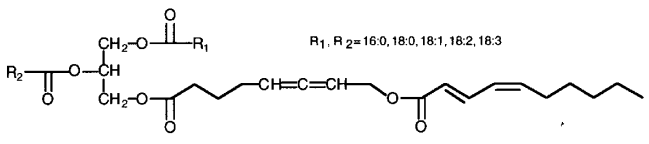
Seed oils of the Euphorbiaceae show a great variability in their lipid composition, and various unusual fatty acid derivatives and other lipid structures have been isolated (1). The seed oils from some members of this plant family, e.g., *Ricinus communis, Vernonia* spp. and *Aleurites* spp., play an important economic role in many countries and are used widely in pharmaceutical and technical applications (2).

Recently, we described the identification of α-parinaric acid from the seed oil of *Sebastiana brasiliensis* (3) and the occurrence of high amounts of vernolic acid in *Bernardia pulchella* (4), both from the Euphorbiaceae family. To extend the knowledge about seed oils of this plant family, we examined a sample of *S. commersoniana* seeds. This plant is a shrub or tree of 2–15-m height, which occurs in Brazil, Uruguay, Argentina, Paraguay, and Bolivia. Its wood is used as firewood, for house construction, and for making agricultural tools. In addition, the roots are considered medicinal (5). Each fruit of this plant contains three oily seeds, which were investigated as a part of our current research project about new seed oils and plant fatty acids from Brazil.

This paper deals with the spectroscopic structure elucidation of the estolide tetraester triglyceride fraction of the title plant, which contains 8-hydroxy-5,6-octadienoic acid and *trans*-2,*cis*-4-decadienoic acid besides other common fatty acids (Scheme 1).

This rare lipid component has already been found in seed oils from the Euphorbiaceae species *Sapium sebiferum* (6) and *Sebastiana ligustrina* (7). The authors (6,7) characterized the individual fatty acids and their linkage to the glycerol moiety by a combination of various chemical, chromatographic, and spectroscopic methods. In brief, the allenic acid was identified by gas chromatography (GC) and by ultraviolet (UV), infrared (IR), and ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy after reduction and acetylation to the corresponding 1,8-octanediol diacetate and 2,3-octadiene-1,8-diol diacetate, respectively. The conjugated *trans*-2,*cis*-4-decadienoic acid was characterized by its UV, IR, and mass spectral data. The linkage of these two rare fatty acids in the tetraester was proven by interpretation of the mass spectrum acquired from the hydrogenated tetraester and by interpretation of the IR and mass spectroscopic data obtained from the individual fractions after incomplete interesterification of the original tetraester.

In the present work, the allene-containing estolide tetraester triglyceride of the seed oil from *S. commersoniana* was elucidated by a combination of UV, IR, fast atom



SCHEME 1

[1]This work was presented in part at the 87th Annual Meeting of the American Oil Chemists' Society, April 28–May 1, 1996, Indianapolis, Indiana.

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Abbreviations: APT, attached proton test; BB, broad band; COSY, correlation spectroscopy; FAB, fast atom bombardment; FAME, fatty acid methyl ester; GC, gas chromatography; IR, infrared; M, molecular ion; MS, mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, trimethylsilyl; UV, ultraviolet; WCOT, wall coated open tubular.

bombardment mass spectrometry (FAB–MS), ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H-correlation spectroscopy (COSY), and gas chromatography–MS (GC–MS). This is the first report on the chemistry of *S. commersoniana*.

MATERIALS AND METHODS

Seed material and oil extraction. The fruits from *S. commersoniana* were collected and botanically identified by Marcos Sobral in January 1995 in the National Park of Turvo, Derrubadas, State Rio Grande do Sul, South Brazil. For extraction of the oil, the air-dried seeds were repeatedly crushed and homogenized with a pestle in a mortar under petroleum ether (40–60°C). After filtration, the solvent was removed by vacuum distillation at 30°C, flushed with nitrogen, and the residue was stored in petroleum ether solution at −18°C until use. The yield was 19.7% of a yellow oil. To minimize autoxidation, all solvents were bubbled with nitrogen before use and contained 0.001% butylated hydroxytoluene (BHT).

Derivatization reactions. Transesterification to the fatty acid methyl esters (FAME) was carried out with 0.5 N sodium methoxide in anhydrous methanol at room temperature under nitrogen as described by Christie (8). Silanization of the FAME mixture was made with bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) (9).

Separation of the estolide and triglyceride fraction. Preparative separation of the seed oil components was done with 0.6-mm preparative silica thin-layer chromatography (TLC) using petroleum ether/diethyl ether (80:20; vol/vol) as solvent system. Detection of the individual fractions was carried out under UV light at 254 nm and after spraying with 2′,7′-dichlorofluorescein under UV light at 340 nm. The TLC bands of interest were scraped off, eluted with diethyl ether, and further analyzed.

GC and GC–MS analyses. A Hewlett-Packard (Palo Alto, CA) 5890 GC Series II GC, a flame-ionization detector (230°C) and a split injector (1:50, 230°C) with glass insert and H_2 as carrier gas (head pressure 1.5 bar) were used to analyze the FAME. Separation of the compounds was achieved with an HP-20M (Hewlett-Packard) capillary column (50 m × 0.2 mm × 0.2 μm), using an oven temperature of 190°C. Peak integration was done with an HP3392A integrator (Hewlett-Packard). For estimation of the equivalent chain length values, the retention times were measured from the time of elution of the solvent, considered as unretained solute. GC–MS was done with the NERMAG AUTOMASS (France, Paris) operating with an ionization energy of 70 eV, a source temperature of 225°C, and an interface temperature of 220°C. The separation of the FAME derivatives was carried out with a BPX-70 (SGE, Weiterstadt, Germany) WCOT column (30 m × 0.22 mm × 0.25 μm) using a temperature program (160–230°C; 2°C/min). Helium was used as carrier gas (0.5 bar).

Spectroscopic procedures. IR spectra were obtained with a Shimadzu FTIR-8101 (Tokyo, Japan) instrument in a liquid film. UV spectra were recorded from 400–200 nm in cyclohexane solution using a Shimadzu UV-2201 spectrophotometer. The ^{1}H NMR, ^{1}H-^{1}H two-dimensional shift correlation (COSY) and ^{13}C NMR broad-band (BB) decoupled and attached proton test (APT) experiments were done on a Bruker AMX500 (Karlsruhe, Germany) spectrometer in deuterochloroform solution. Preliminary NMR data were obtained with a Varian VLX-200 (Palo Alto, CA) spectrometer with trimethylsilyl (TMS) as internal standard. FAB(+) mass spectra of the allene estolide were measured with a VG analytical ZAB-HF reversed geometry mass spectrometer (Manchester, United Kingdom) with xenon as bombardment gas. A mixture of 1-mercapto-2,3-propandiol and *meta*-nitrobenzylalcohol (1:1) was used as matrix, and sodium acetate was added. Spectra were recorded and evaluated on SAM II/68 K computer (KWS, Ettlingen, Germany) using the DP10 program from AMD (Harpstedt, Germany).

RESULTS AND DISCUSSION

Preliminary experiments. TLC analysis of the seed oil of *S. commersoniana* showed two main components (FR1: R_f = 0.78 and FR2: R_f = 0.54) along with other minor spots. FR1 showed the same migration characteristics as authentic triglycerides from sunflower oil. FR2 is generally not detectable in common seed oils and was the only component of the lipid mixture that was directly detectable under UV light at 254 nm. The UV spectrum of the oil showed a maximum at 260 nm and the other spectral data (IR, ^{1}H and ^{13}C NMR) also presented some characteristics which were not observed in common seed oils. Consequently, the two main fractions, FR1 (69%) and FR2 (31%), were isolated by preparative TLC and further examined.

Characterization of FR1. The IR, ^{1}H, and ^{13}C NMR data of FR1 showed the same features as triglycerides containing saturated and olefinic (oleic, linoleic, and linolenic acids) fatty acids. No UV absorption could be detected. These observations were in good agreement with the TLC data, suggesting that FR1 was a simple triglyceride fraction. The fatty acids of this fraction were analyzed after transmethylation by GC and GC–MS, and the results are summarized in Table 1. Linoleic (16%) and linolenic acid (68%) were found to be the main compounds. Identification of the fatty acids was made by co-injection of standard compounds and by comparison of the mass spectra with the spectra of standards.

Characterization of FR2. The UV spectrum of FR2 showed a broad absorption at 260 nm, attributable to the presence of carboxyl-conjugated fatty acids (6). The IR spectrum showed, in addition to the triglyceride bands, absorption peaks at 1717 cm^{-1} (s), 1635 cm^{-1} (m), 997 cm^{-1} (m), and 870 cm^{-1} (m), pointing to a conjugated *trans*-2,*cis*-4-dienoate system (10). A *cis*-2,*trans*-4-dienoate structure was excluded since its characteristic bands at 971 and 820 cm^{-1} did not appear. The band at 1966 cm^{-1} (w) in the IR spectrum was assigned to an allene structure (6).

GC and GC–MS analysis of the FAME of FR2 indicated the presence of common fatty acids with α-linolenic acid as

TABLE 1
Fatty Acid Composition (wt%, uncorrected) of the Triglyceride (FR1) and Estolide Fractions (FR2) from *Sebastiana commersoniana*

Fatty acid	FR1	FR2
?	—	0.2
10:2(2*t*, 4*c*)	—	9.9
10:2 (2*t*, 4*t*)?	—	1.4
14:0	0.23	—
?	0.61	0.2
?	—	0.1
16:0	4.2	31.2
8-OH-8:2(5,6)	—	4.7
18:0	3.25	2.4
18:1n-9	7.2	3.8
18:1n-7	0.41	0.3
18:2n-6	15.64	9.3
18:3n-3	67.61	36.2
20:0	0.25	0.1
20:1n-9	0.60	0.3

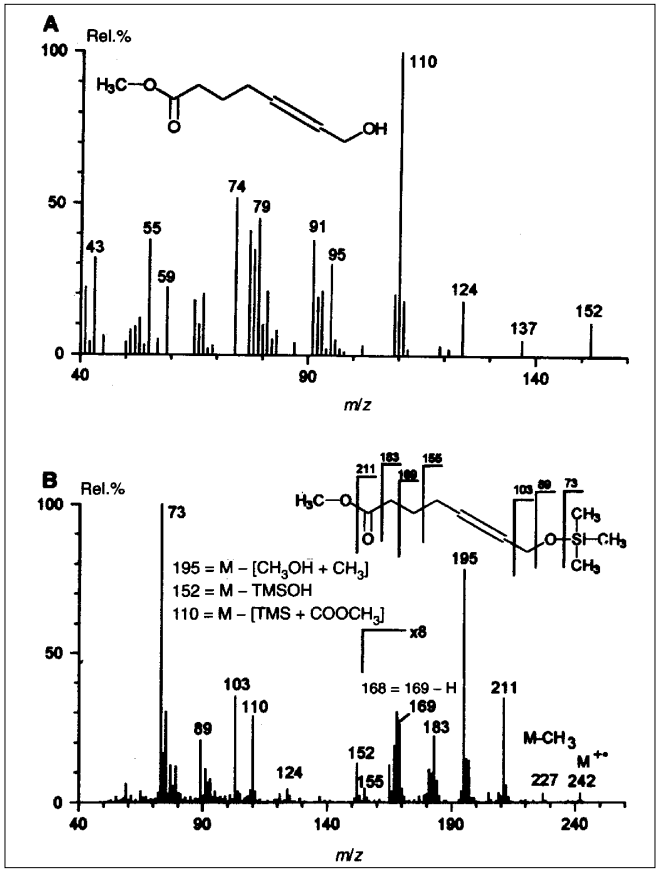


FIG. 1. The mass spectra of the methyl esters of 8-hydroxy-5,6-octadienoic acid [A = with free OH group, B = as its trimethylsilyl (TMS) derivative)].

the main fatty acid (Table 1). Furthermore, three GC peaks (P1a, P1b, and P2), which showed retention characteristics not usually found in seed oil fatty acids, were observed. The mass spectrum of P1a (equivalent chain length = 11.96) showed a molecular ion at *m/z* 182, suggesting the molecular formula $C_{11}H_{18}O_2$. The fragmentation pattern was identical to that published for deca-2,4-dienoic acid methyl ester (10). Taken together, the MS data and the UV, IR, and ^{13}C NMR data from FR2 were consistent with P1 being identified as 2-*trans*,4-*cis*-decadienoic acid methyl ester. The minor compound P1b (equivalent chain length = 12.4) appeared to be a stereoisomer of P1a since both compounds showed the same mass spectrum. The peak at equivalent chain length 16.65 (P2) disappeared after silanization, and instead, appeared as a new peak eluting between P1 and palmitic acid methyl ester. This behavior suggested that compound P2 contained a free hydroxyl group. The mass spectrum of P2 (Fig. 1), subsequently identified as 8-hydroxy-5,6-octadienoic acid methyl ester, was not very useful for further structure assignments. The peak with the highest mass value was observed at *m/z* 152, which was attributed to the loss of H_2O from the molecular ion at 170 mu ($C_9H_{14}O_3$). The base peak at *m/z* 110 was explained by the loss of the methyl ester moiety and simultaneous rearrangement of one hydrogen atom ($M - CH_3OCOH$?). In the low-mass range, the characteristic methyl ester ions (*m/z* 59, 74) were detected, and the highly unsaturated hydrocarbon fragments (*m/z* 41, 55, 79, 91, etc.) suggested that rearrangements occurred during ionization.

The fragmentation pattern of silylated P2 (Fig. 1) provided more structural information. The rearrangement reactions were suppressed in the silylated derivative. Suggested fragmentation ions are included in the mass spectrum of silylated P2 (Fig. 1). In the low-mass range, the ion series containing the TMS moiety (*m/z* 73, 89, and 103) appeared with abundance and *m/z* 73 was the base peak. The gap between the ions *m/z* 103 and 155 was diagnostic for the location of the allenic system, since direct cleavage at the double-bond system is energetically unfavorable. The ion series, containing the TMS moiety, was continued at *m/z* 155, 169, 183, and represented the successive cleavage at each methylene group beginning from the allylic methylene group in the direction of the methyl ester end (Fig. 1B). The weak signal at *m/z* 242 was attributed to the molecular ion, confirmed by the ions at *m/z* 227 ($M - CH_3$) and 211 ($M - OCH_3$). Further confirmation on the structure of P2 were obtained by the NMR data (see below). Since the IR spectrum did not show any absorption for a free hydroxy function, it was concluded that the hydroxy group was linked to another fatty acid in FR2.

The mass spectra of 8-hydroxy-5,6-octadienoic acid methyl ester and its corresponding TMS derivative have not been previously published. The mass spectrum published for the acetate of 8-hydroxy-5,6-octadienoic acid methyl ester (12) did not show clearly the molecular ion and was dominated by the $[COCH_3]^+$ fragment (*m/z* 43). The remaining fragmentation pattern of the published spectrum (12) was essentially similar to the nonacetylated derivative in this study (Fig. 1A), but the position of the allenic system had not been investigated previously (12).

The GC analysis of 8-hydroxy-5,6-octadienoic acid methyl ester was described as problematic in the literature. Sprecher *et al.* (6) mentioned that this compound was proba-

bly not detected during GC analysis because polymerization or decomposition of the highly reactive allylic alcohol may have occurred on the GC column. Similar observations were made by other research groups (7,13) who used packed GC columns for analysis. The gas chromatograms obtained with a Silar 5 CB WCOT capillary column from stillingia oil fractions (14), which also contained 8-hydroxy-5,6-octadienoic acid, did not show any evidence of this allenic fatty acid. The authors used BF_3/methanol for transesterification, and the C_8 fatty acid may have undergone side reactions during derivatization. Acidic conditions are generally not recommended for the transesterification of unusual fatty acids (8). The results from our GC analysis of the methyl esters from FR2 showed that 8-hydroxy-5,6-octadienoic acid was detected by GC by using mild transesterification conditions and a Carbowax 20M WCOT column. However, the integrated value of P2 demonstrated (Table 1) that the recovery may not be quantitative, and some decomposition may have occurred.

To obtain more detailed structure information, the FAB, MS, and NMR spectra of FR2 were studied. The quasi-molecular ions $[M + Na]^+$ in the range from *m/z* 901 to 929 in the FAB mass spectrum (Fig. 2 and Table 2) were compatible with a triglyceride structure that contained two mols of common saturated and unsaturated C_{16} and C_{18} fatty acids, one mol of 2-*trans*,4-*cis*-decadienoic, and one mol of 8-hydroxy-5,6-octadienoic acid. The peaks in the range from *m/z* 573 to 601 (E_2) and the peak at *m/z* 289 (R_3) showed clearly that 8-hydroxy-5,6-octadienoic acid was esterfied with 2-*trans*,4-*cis*-decadienoic acid, resulting in a "C_{18} chain." No ions that could prove a connectivity between 8-hydroxy-5,6-octadienoic acid and any one of the C_{16} and C_{18} fatty acids were observable. The observation of a base peak at *m/z* 151, derived from acyl cleavage from 2-*trans*,4-*cis*-decadienoic acid $[^+CO–CH=CH–CH=CH–(CH_2)_4CH_3]$, without accompanying fragments at *m/z* 151 + 74 and *m/z* 151 + 128, respectively, showed that this conjugated fatty acid was not directly linked to the glyceride moiety, but was esterfied to the hydroxyl group to 8-hydroxy-5,6-octadienoic acid. It is known that in triglycerides the fragments due to a cleavage at the acyl group are accompanied by peaks with mass numbers 74 and 128 units higher than each original acyl group. These ions show the connectivity for a fatty acid with the glyceride backbone (6) and were detected here for linoleic acid at *m/z* 261 (RCO^+), *m/z* 335 (261 + 74), and *m/z* 389 (261 + 128) in the mass spectrum. The explanations for the main fragments of the FAB mass spectrum of FR2 are summarized in Table 2. Unfortunately, these mass spectral data did not provide information whether the "C_{18} chain," containing 2-*trans*,4-*cis*-decadienoic acid and 8-hydroxy-5,6-octadienoic acid, was linked to the α- or β-hydroxy group of the glycerol moiety.

By careful interpretation of the NMR data, the structure elucidation of FR2 was completed. Comparison with standard compounds and literature data (15) permitted the assignment of most ^1H and ^{13}C NMR signals of FR2 (Tables 2 and 3). The presence of saturated and unsaturated fatty acids linked to a glyceride backbone was clearly evident. The assignment of the remaining signals was made in conjunction with ^1H NMR, ^1H-^1H two-dimensional shift correlation (COSY), ^{13}C NMR (BB decoupled and APT mode) spectroscopic experiments and by comparison with literature data.

The ^1H-^1H COSY NMR spectra (Fig. 3) were consistent with the identification of 8-hydroxy-5,6-octadienoic acid, made by GC–MS analysis of its TMS derivative: The triplet at δ 2.35 ppm showed a cross signal with the multiplet at δ 1.75 ppm. The latter signal was further correlated with the signal at δ 2.05 ppm that also showed a cross signal with δ 5.3 ppm. These correlation patterns were compatible with a partial structure $ROOC–CH_2–CH_2–CH_2–CH=$. The signal at

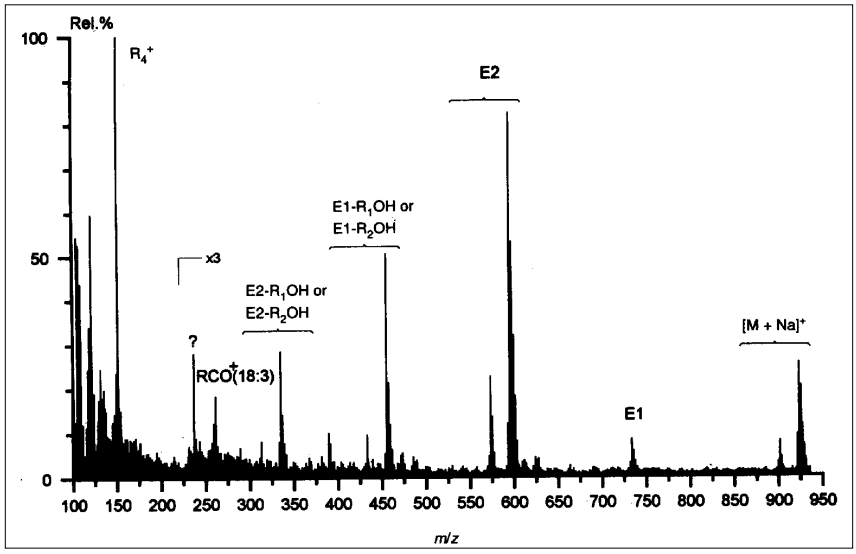
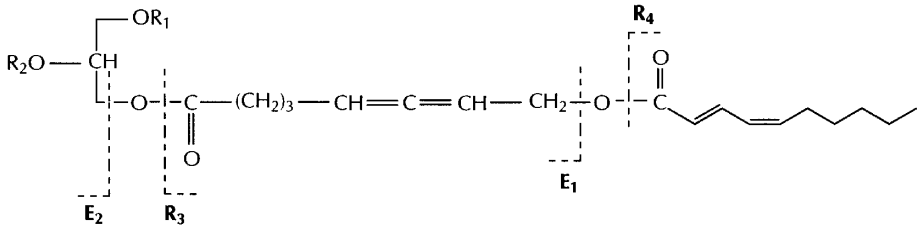


FIG. 2. The fast atom bombardment mass spectrum of the estolide fraction FR2 of the seed oil from *Sebastiana commersoniana*. The indicated letters correspond with those in Table 2.

TABLE 2
The Fast Atom Bombardment Mass Spectral Data of the Allene Estolide Fraction (FR2) from *Sebastiana commersoniana* (R_1 and R_2 are interchangeable)



m/z	Ion	R_1	R_2	*m/z*	Ion	R_1	R_2
929	$[M + Na]^+$	18:0	18:3	461	E_1-R_1OH or E_1-R_2OH	18:0	—
927	$[M + Na]^+$	18:1	18:3	459	E_1-R_1OH or E_1-R_2OH	18:1	—
925	$[M + Na]^+$	18:2	18:3	457	E_1-R_1OH or E_1-R_2OH	18:2	—
923	$[M + Na]^+$	18:3	18:3	455	E_1-R_1OH or E_1-R_2OH	18:3	—
903	$[M + Na]^+$	18:2	16:0	433	E_1-R_1OH or E_1-R_2OH	—	16:0
901	$[M + Na]^+$	18:3	16:0	395	R_1O–CH=CH–CH_2–O–CO–CH_2–CH_2[1] ?	18:0	—
739	E_1	18:0	18:3	393	R_1O–CH=CH–CH_2–O–CO–CH_2–CH_2[1] ?	18:1	—
737	E_1	18:1	18:3	391	R_1O–CH=CH–CH_2–O–CO–CH_2–CH_2[1] ?	18:2	—
735	E_1	18:2	18:3	389	R_1O–CH=CH–CH_2–O–CO–CH_2–CH_2[1] ?	18:3	—
733	E_1	18:3	18:3	341	E_2-R_1OH or E_2-R_2OH	18:0	—
629	$[MH]^+ - R_2OH$	18:0	—	339	E_2-R_1OH or E_2-R_2OH	18:1	—
627	$[MH]^+ - R_2OH$	18:1	—	337	E_2-R_1OH or E_2-R_2OH	18:2	—
625	$[MH]^+ - R_2OH$	18:2	—	335	E_2-R_1OH or E_2-R_2OH	18:3	—
623	$[MH]^+ - R_2OH$	18:3	—	313	E_2-R_1OH or E_2-R_2OH	—	16:0
601	E_2	18:0	18:3	289	R_3^+		
599	E_2	18:1	18:3	261	RCO^+ (18:3)		
597	E_2	18:2	18:3	237	?		
595	E_2	18:3	18:3	151	R_4^+		
577	E_2	18:1	16:0				
575	E_2	18:2	16:0				
573	E_2	18:3	16:0				

δ 4.6 ppm revealed only one cross signal with δ 5.3 ppm. In the 200 MHz ^{1}H NMR spectrum (Fig. 4) the signal at δ 4.6 ppm appeared as a double doublet (*dd*, *J* = 6.6, 2.2 Hz), suggesting a –CH=C=CH–C*H*$_2$–O–R structure. Obviously, the coupling of the CH_2–O–R protons extended up to the methylene protons at the other side of the allenic system, since in the 500 MHz ^{1}H NMR spectrum (Fig. 4) the signal at δ 4.6 ppm was split into a more complex multiplet. The theoretically possible 2,3-, 3,4-, 4,5-, and 6,7-octadienoic structures were excluded by the interpretation of the ^{1}H-^{1}H COSY data, and by comparison of the ^{1}H NMR shifts from synthetic positional isomers of methyl allenic fatty acids (16). The ^{1}H integral value of the signal at δ 4.6 ppm was identical to the integral value for the signals of the α-methylene protons from the glyceride backbone at δ 4.15 and 4.3 ppm. This observation demonstrated that the α-methylene protons from the glyceride backbone were present in the same molecular proportion as the methylene protons from the =CH–C*H*$_2$–O–R linkage. In agreement with the IR and FAB MS data, no signals for a free OH group were detected in the NMR spectra. The ^{1}H NMR signals of the *trans* carboxyl-conjugated system were observed at δ 5.85 ppm (2H, *m*), δ 6.15 ppm (1H, *dd*, *J* = 11,11 Hz) and δ 7.62 ppm (1H, *dd*, *J* = 15,11 Hz). The coupling constants, the multiplicity pattern, and the ^{1}H-^{1}H COSY correlation sequence (δ 5.85, 7.62, 6.15 ppm) were in agreement with the conjugated *trans*-2,*cis*-4-dienoate system.

TABLE 3
^{1}H Nuclear Magnetic Resonance Shifts (in ppm) of the Estolide Fraction from *Sebastiana commersoniana* (FR2)[a]

Signal	Assignment
0.88 (*t*)	C*H*$_3$– (S, M, L)
0.97 (*t*)	C*H*$_3$– (Ln)
1.28 (*m*)	–(C*H*$_2$)$_n$– (all FA)
1.6 (*m*)	C*H*$_2$--CH$_2$–COOR (all FA)
1.75 (*m*)	C*H*$_2$--CH$_2$–COOR (Al)
2.05 (*m*)	–CH=CH–C*H*$_2$–
2.3 (*t*)	–C*H*$_2$–COOR (all FA)
2.35 (*t*)	–C*H*$_2$–COOR (Al)
2.8 (*m*)	–CH=CH–C*H*$_2$–CH=CH– (L,Ln)
4.62 (*m*)	–CH=C=CH–C*H*$_2$–O–COR (Al)
5.3 (*m*)	–C*H*=C*H*– (M,L,Ln), –C*H*=C=C*H*– (Al)
5.85 (*m*)	–*trans*-C*H*=CH–*cis*-CH=C*H*– (Con)
6.18 (*dd*; *J* = 11;11 Hz)	–*trans*-CH=CH–*cis*-C*H*=CH– (Con)
7.62 (*dd*; *J* = 15;11 Hz)	–*trans*-CH=C*H*–*cis*-CH=CH– (Con)

[a]FA, fatty acids, S, saturates; M, monoenes; L, linoleate; Ln, linolenate; Al, 8-hydroxy-5,6-octadienoate; Con, *trans*-2,*cis*-decadienoate.

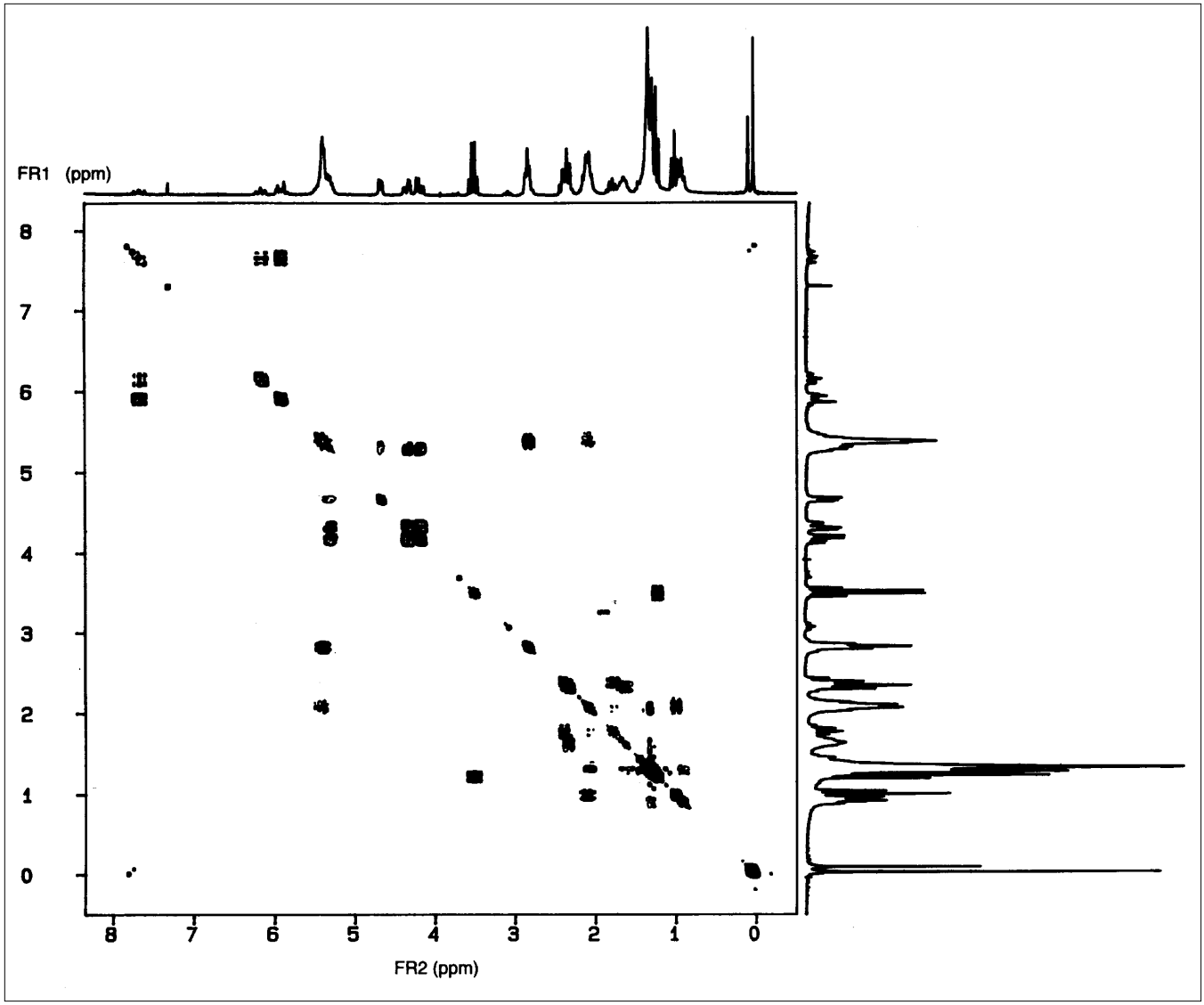


FIG. 3. The ^{1}H-^{1}H COSY NMR spectrum (200 MHz) of the estolide fraction FR2 of the seed oil from *Sebastiana commersoniana*.

Similar ^{1}H NMR data were observed for a comparable ketodiene structure in 13-keto-9-*cis*-11,*trans*-octadienoic acid methyl ester (17).

The ^{13}C spectra [BB decoupled (Fig. 5) and ATP mode] provided information about the linkage of the estolide part at the glyceride backbone (Table 4). In the range between δ 62 and 69 ppm, three –*C*H$_2$O– signals (δ 62.15, 62.3, and 62.6 ppm) and one –*C*HO– signal (δ 68.93 ppm) appeared with nearly identical intensity. Two of the *C*H$_2$O– signals were attributed to the two α-hydroxy carbons from glycerol, and the third *C*H$_2$O– signal was assigned to –CH=C=CH–*C*H$_2$O– of the estolide. In normal triglycerides, only one –*C*HO– signal and one double intense *C*H$_2$O– signal for the two CH$_2$ groups are observed (15). The fact that the *C*H$_2$O– signals for the two glyceride CH$_2$ groups were resolved into two signals was due to the influence of the allenic double bond system at C-5 of the estolide. This observation suggests that the estolide was linked to a α-hydroxy group of the glyceride moiety. If the estolide was linked to the β-hydroxy group of glycerol, no splitting of the two glyceride CH$_2$ groups would be expected. The possibility that FR2 was a mixture of triglycerides with the estolide connected to the α- and β-position of the glycerol moiety was excluded by the equal intensity of the three signals between δ 62 and 69 ppm.

The signals for the allenic double-bond system at δ 205.51 ppm (–CH=*C*=CH–), δ 87.76 ppm (–CH=C=*C*H–CH$_2$–O–R) and δ 91.95 ppm (–*C*H=C=CH–CH$_2$–O–R) were assigned by comparison with data from a synthetic allenic C$_{18}$ fatty acid methyl ester (16). The position of the double bonds in the hydrocarbon chain of the allenic fatty acid was confirmed by the comparison with the ^{13}C NMR data of 5,6-octadecadienoic acid methyl ester (16). The observed signals at δ 33.27, 24.1, 28.34 ppm were assigned to C$_2$ to C$_4$ in good agreement with the ^{13}C signals of C$_2$ to C$_4$ of the latter compound (16). It was

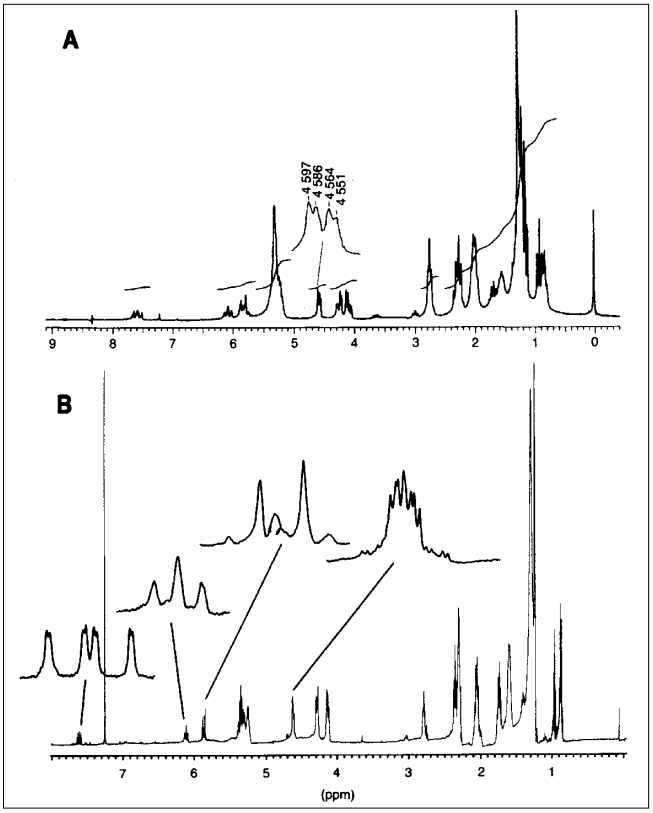


FIG. 4. The ^{1}H NMR spectrum (200 (A) and 500 MHz (B)) of the estolide fraction FR2 of the seed oil from *Sebastiana commersoniana*.

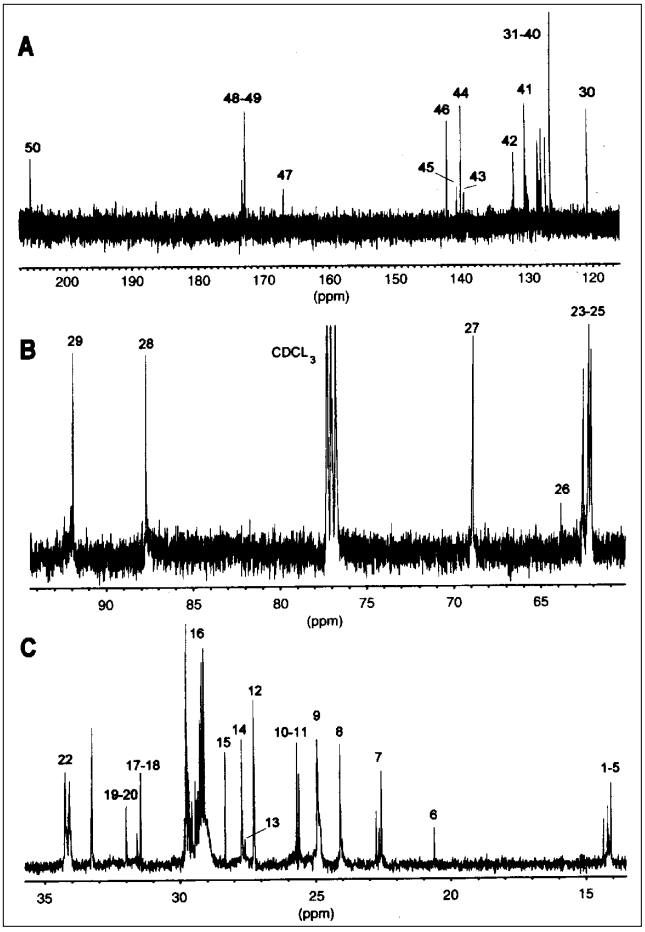


FIG. 5. The ^{13}C NMR spectrum [125 MHz (B)]) of the estolide fraction FR2 of the seed oil from *Sebastiana commersoniana* (A = 120–210 ppm; B = 60–94 ppm; C = 14–35 ppm). The peak numbers correspond with Table 4.

shown recently that ^{13}C NMR data permitted the characterization of 11 of 15 positional isomers of synthetic allenic C_{18} FAME (16). The remaining ^{13}C NMR signals were assigned to the 2-*trans*,4-*cis*-decadienoic acid part of FR2. The signal at δ 167.07 ppm was due to the carbonyl group in conjugation with the double bonds (18). The only signals in the ^{13}C NMR spectra that could not be assigned directly by data comparison appeared at δ 120.83, 126.47, 140.06 and 142.09 ppm, and could be due to the four conjugated double-bond carbons. Some minor signals in the double-bond range (δ 139.6 and 140.65 ppm) may be due to the olefinic carbons of the stereoisomer of 2-*trans*,4-*cis*-decadienoic acid detected in the GC–MS analysis.

In conclusion, interpretation of the combined spectroscopic and chromatographic data allowed for the identification of FR2 from *S. commersoniana* as a tetraester triglyceride composed of the allenic hydroxy acid 8-hydroxy-5,6-octadienoic acid jointed in an estolide linkage to 2-*trans*,4-*cis*-decadienoic acid. The same type of compound has already been isolated from *S. sebiferum* (6) and *S. ligustrina* (7). Christie (13) showed that the estolide from *S. sebiferum* occurs exclusively in the *sn*-3-position of the glyceride moiety and suggested that the optical activity of this molecule is mainly caused by the allenic system. The optical activity of the seed oil from *S. commersoniana* was not carried out in this study.

From a chemotaxonomic point of view, it is interesting to note that this estolide compound was not detected in the related species *S. brasiliensis* (3) and *Stillingia sylvatica* (7). In a preliminary analysis, it was shown that the seed oil of *Sapium japonicum* contained the same IR bands which had the same TLC and high-performance liquid chromatography properties as the allene triglyceride from *S. sebiferum* (19). A fraction isolated from *Stillingia texana* stem lipids also showed IR and mass spectroscopic evidences that a substance with some relationship to the allene estolide (7) was present.

The physiological functions and the biosynthesis of this rare estolide structure are unexplored. Recently, it was shown that 8-hydroxy-5,6-octadienoate methyl ester, isolated from the leaves of *S. japonicum*, has antifungal properties (12). Stillingia oil from *S. sebiferum* is considered nutritionally undesirable (20). The seed oil from *S. commersoniana* also can not be recommended for human diet. The high content of linolenic acid and the presence of the unstable estolides classifies the oil as a good drying oil.

TABLE 4
^{13}C Chemical Shifts (in ppm relative to TMS) of the Estolide Fraction (FR2) from *Sebastiana commersoniana* and Their Assignments[a]

Signal	^{13}C-Shift	Assignments[b]	Signal	^{13}C shift	Assignments
1	13.81 (weak)	CH_3– (?)	26	63.88 (weak)	?
2	14.09	CH_3– (S, M)	27	68.93	β-Gly
3	14.16	CH_3– (L)	28	87.76	–CH=C=*C*H–CH_2–O–COO– (Al)
4	14.20	CH_3– (10:2) ?	29	91.95	–*C*H=C=CH–CH_2–O–COO– (Al)
5	14.36	CH_3– (Ln)	30	120.83	*trans*-CH=CH–*cis*-CH=CH– (Con)
6	20.63	C-17 (Ln)	31	126.47	*trans*-CH=CH–*cis*-CH=CH– (Con)
7	22.57/22.65/22.77	CH_3–CH_2–	32	127.19	C-15 (Ln)
8	24.1	C-3 (8-OH-8:2)	33	127.84/127.86	C-10 (Ln; α/β-glyceride)
9	24.91/24.94	C-3 (S, M, L, Ln)	34	127.97	C-12 (L)
10	25.61	C-14 (Ln)	35	128.15	C-10 (L)
11	25.7	C-11 (L, Ln)	36	128.31	C-12 (Ln)
12	27.28	–CH=CH–CH_2–CH_2-(M, C-8: L, Ln)	37	128.38	C-13 (Ln)
13	27.61 (weak)	allyl CH_2 ?	38	129.76/129.78	C-9 (O; α/β-glyceride)
14	27.73	*trans*-CH=CH–*cis*-CH=CH–*C*H_2– (Con)	39	130.04/130.09	C-10 (O), C-9 (L)
15	28.34	C-4 (Al)	40	130.28	C-13 (L)
16	29.12-29.85	–$(CH_2)_n$– (all FA)	41	130.31	C-9 (Ln)
17	31.46	CH_3–CH_2–*C*H_2– (Con) ?	42	132.04	C-16 (Ln)
18	31.6	CH_3–CH_2–*C*H_2– (L)	43	139.58 (weak)	?
19	31.98	CH_3–CH_2–*C*H_2– (M)	44	140.06	*trans*-CH=CH–*cis*-CH=CH– (Con)
20	32.00	CH_3–CH_2–CH_2– (S)	45	140.65 (weak)	?
21	33.27	C-2 (Al)	46	142.09	*trans*-CH=CH–*cis*-CH=CH– (Con)
22	34.09-34.25	C-2 (S, M, L, Ln, P)	47	167.07	–COOR (Al)
23	62.15	α-Gly (linked with usual FA)	48	172.94	–COOR (R = Al at α-Gly); –COOR at β-Gly; R = usual FA
24	62.30[b]	α-Gly (linked with Al)	49	173.34/173.40	–COOR at α-Gly; R = usual FA
25	62.60[b]	–CH=C=CH–CH_2–O–COOR (Al)	50	205.51	–CH=*C*=CH– (Al)

[a]Abbreviations: FA, fatty acids; Gly, glycerol carbons; S, saturates; M, monoenes; L, linoleate; Ln, linolenate; Al, 8-OH-5,6-octadienoate; Con, *trans*-2,*cis*-decadienoate. [b]Assignment signals may be reversed.

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Nonionic Surfactants. Regioselective Synthesis of Fatty Acid Esters of α- and β-Glucopyranose

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ABSTRACT: Lipophilic esters of saccharides belong to the family of nonionic surfactants widely employed in pharmaceutical and cosmetics formulations. A very simple method is presented whereby 6-*O*-esters of α- and β-glucose can be prepared and isolated. Good results have been obtained in the synthesis of 6-*O*-oleyl derivatives by simple acylation with appropriate oleyl chloride. The condensing agent bis(2-oxo-3-oxazolidinyl) phosphinic chloride (BOP-Cl) allows the preparations of the same esters with better regioselectivity but with yields strongly dependent on the aliphatic chain length of the carboxylic acids employed.

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Surfactants are molecules which exert a wide range of biological, pharmacological, and toxicological activity. The role of nonionic surfactants in drug delivery systems has received an enormous interest in recent years (1) since they are generally regarded as most suitable for pharmaceutical formulation (2). On the other hand, polyglycosides bearing alkyl chains are nowadays of paramount technological interest as moderately foaming material to be used as additives in cleaning products (3) or in cosmetics (4). Biocompatible nonionic surfactants can be obtained by linking a hydrophobic alkyl chain to a polyhydroxylated system, usually a carbohydrate molecule, through ester linkages (5–7). The surface-active behavior of these esters depends on the length of the lipophilic chain, the temperature, and the position of the ester group in the hydrophilic head of the sugar (7,8). The selected acylation of one out of the multiple hydroxyl groups present in a sugar substrate has been achieved by means of tributylstannosides (9), cobalt chelates (10), Mitsunobu reaction (11–13), and by using anhydrides and condensing agents (14,15). In particular, it has been recently demonstrated that bis(2-oxo-3-oxazolidinyl) phosphinic chloride (BOP-Cl) (16), a widely employed condensing agent (16–23), provides valuable and simpler alternatives to enzymatic procedures (24–30) in the regioselective esterification of primary (17) hydroxyl groups of both gluco- and mannopyranosides. Unfortunately, the straightforward esterification of saccharides with acyl chlorides leads to mixtures of mono- and polysubstituted derivatives which are often difficult to separate (31,32). Nonetheless the need of producing new surfactants with a high degree of purity and very simple chemistry has prompted the exploitation of the regioselective acylation of 1,2-isopropylidene-D-glucofuranose (**1**) with acyl chlorides or with correspondent carboxylic acids activated by means of BOP-Cl.

The 1,2-isopropylidene-D-glucofuranose **1** (Scheme 1) has already been used, in connection with a series of reactions, to introduce new functional groups into the D-glucopyranose ring (33,34). Although it is commercially available, **1** may be readily prepared from the less expensive 1,2:5,6-di-*O*-isopropylidene-D-glucofuranose by preferential hydrolysis of the more acid-labile 5,6-isopropylidene group (34). The furanose **1** therefore has been chosen as a suitable substrate in the formation of nonionic surfactants in the glucopyranose series, since it provides means for a facile deprotection of the anomeric hydroxyl group at the end of the synthesis.

RESULTS AND DISCUSSION

The reactivity of the free hydroxyl groups in compound **1** has been exploited with two independent acylating procedures (Scheme 1): (i) the conventional treatment with acyl chlorides **2a–d** (Method A) and (ii) the employment of BOP-Cl with carboxylic acids **3a–d** (Method B). The data reported in Table 1 show that method A gives, under the adopted experimental conditions, good to excellent yields of products **4a–d**, owing to the higher reactivity of primary vs. secondary hydroxyl groups of the furanoside. The expected (17) high re-

TABLE 1
Acylation of 1,2-Isopropylidene-D-glucofuranose (compound 1)

	Time (h) (yields)	
Compounds	Method A	Method B
4a	2 (70)	2 (56)
4b	6 (73)	8 (60)
4c	6 (70)	16 (58)
4d	2.5 (93)	24 (15)

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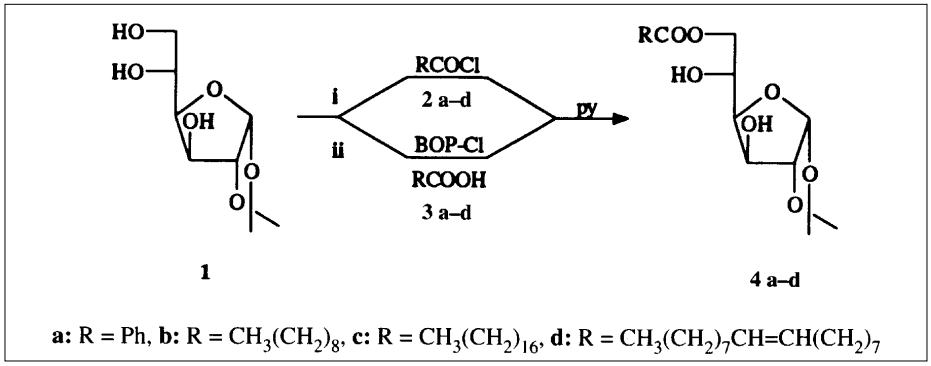
Abbreviations: BOP-Cl, bis(2-oxo-3-oxazolidinyl) phosphinic chloride; FAB, fast-atom bombardment; HPLC, high-performance liquid chromatography; MS, mass spectra; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, trimethylsilyl.



SCHEME 1

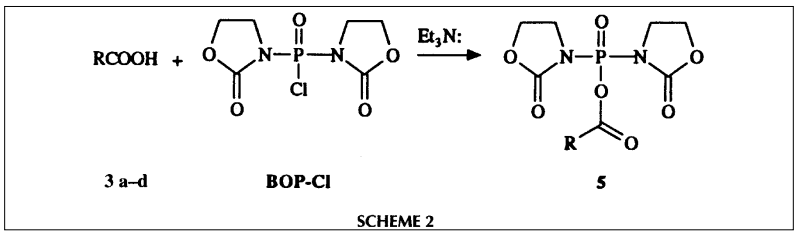
gioselectivity experienced with BOP-Cl was counterbalanced by the low isolated yields of the final product. The reaction of **1** with **2a–d** in pyridine affords mainly the products **4a–d** and other less polar polyacylated derivatives which were not isolated or identified. The adopted experimental conditions (Table 1) represent the best of many trials in terms of conversion-to-selectivity ratio.

When **1** was allowed to react with **3a–c** in the presence of BOP-Cl, the formation of side products was negligible, even though the total conversion was lower than with Method A. In the case of the oleic acid **3d**, a clean product **4d** was obtained, without any purification, at the expense of the total yield. It should be considered that if the goal is the obtainment of pure material the simpler acylation procedure (Method A), followed by chromatographic purification, is the method of choice.

Benzoyl chloride **2a** reacts faster than the aliphatic acyl chlorides **2b**, **c** and with a rate similar to the oleyl derivative **2d**. The latter gives also nearly quantitative yields of a single acylated product **4d**. It can be suggested that the acylation reaction is controlled, in this case, by steric hindrances alone, owing to the reduced number of rotational degrees of freedom of an unsaturated vs. saturated fatty acid chain. When, in fact, some of the rotational degrees of freedom are frozen by the introduction of a *cis*-9,10 double bond into the aliphatic chain, the reactivity of the fatty acid (**2d**) becomes similar to that of **2a**, within the expected experimental error made in determining the end of the reaction. When Method B is used, the acylating agent should likely be the mixed anhydride **5** (16) (Scheme 2). The latter reacts with the hydroxyl groups of the sugar substrate displacing the phosphodiester moiety which is a leaving group as good as the chloride ion (Method A). The kinetics of ester formation therefore might follow the same trend verified when acyl chlorides were used. Conversely, the derivatization of the substrate becomes more difficult on going from **3c** to **3d**. The increasing bulkiness experienced when acids **3a–c** were used could explain, by analogy with similar systems (17), the observed trend. The anomalous behavior of oleic acid (**3d**) might be due to factors, not clearly understood, which prevent the formation of the reacting mixed anhydride (**5**, Scheme 2).

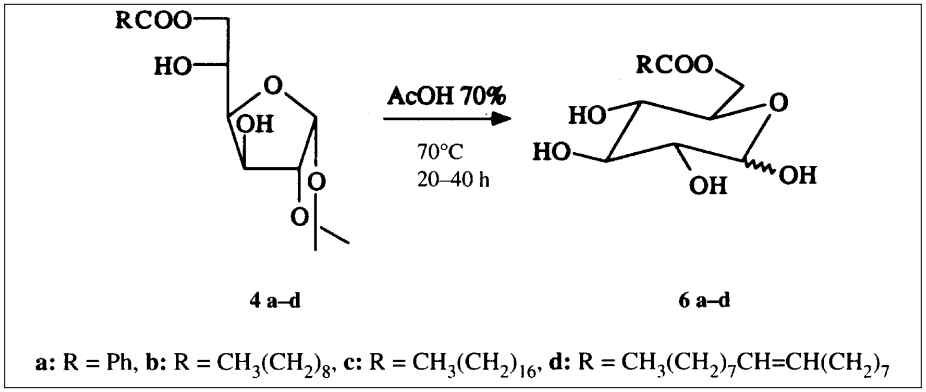
The unprotected target molecules were obtained by removing the 1,2-isopropylidene protecting group in carefully controlled conditions in order to minimize the possible concomitant acid-catalyzed hydrolysis of the ester function.

Trifluoroacetic acid in tetrahydrofuran/water (4:1, vol/vol) at room temperature (8,35) left the molecules unchanged. Treatment of **4a–d** with 1 N sulfuric acid at 100°C did not give satisfactory results, whereas the degradation risk was too high (Scheme 3). The employment of BCl_3 (36) has been rejected to avoid deacylation. Finally the best results have been obtained by means of 70% aqueous acetic acid at 70°C for 20–40 h (Table 2) (37). The pyranoses **6a–d** have been obtained as mixture of α and β anomers, as detected by high-performance liquid chromatography (HPLC) analysis and ^1H nuclear magnetic resonance (NMR) spectroscopy. The lower yields observed in the deblocking of the dioxolane ring when the monosaccharide was esterified with an aliphatic carboxylic acid are due to the concomitant hydrolysis of the ester function which is more acid-labile than in the case of the derivative **4a**.



SCHEME 2

SCHEME 2



SCHEME 3

TABLE 2
Formation of 6a–d by Acid Hydrolysis

Compound	Time (h)	Yield (%)
6a	40	90
6b	24	51
6c	20	50
6d	25	51

The regioselective formation of monosaccharide esters with long-chain fatty acids is considered a difficult task which can be fulfilled by adopting special conditions (27). The results discussed above show a very simple strategy, whereby these surfactant molecules of potential pharmacological and industrial interest can be synthesized by applying very simple chemical methodologies.

EXPERIMENTAL PROCEDURES

BOP-Cl and 1,2-isopropylidene-D-glucofuranose are commercially available or can be easily prepared as previously reported (18–23). Pyridine was distilled from calcium hydride and stored under nitrogen. Precoated silica gel plates were used for thin-layer chromatography (TLC) and Kieselgel 60 H (Merck, Gibbstone, NJ) without gypsum was used for short-column chromatography. HPLC system: BIOLC (Dionex, Sunnyvale, CA), sample loop: 20 µL, column 250 × 4.6 mm, Chromospher C_{18} 5 µm Chrompack; detection: ultraviolet 272 nm, Dionex (variable wavelength). Solvent A, 1% HOAc aq.; B, MeOH; 15% B in A to 30% B in A in 15 min; 30% B in A, 5 min; 30% B in A to 100% B in 5 min; 100% B, 5 min; 100% B to 15% B in A in 5 min; flow, 0.8 mL min^{-1}. NMR spectra were obtained from WM-300 Bruker spectrometer (Karlsruhe, Germany) with tetramethylsilane (TMS) as internal standard and hexadeuterodimethylsulfoxide or trideuteromethyl alcohol as solvents; *J* values are given in Hz. Structural assignments have been made after extensive decoupling experiments. Fast-atom bombardment (FAB)–mass spectra (MS) were obtained on a VG-micromass ZAB 2F mass spectrometer (Manchester, United Kingdom), from 2 mm^3 of *m*-nitrobenzyl alcohol or glycerol solution of sample by using the standard gun operated with neutral xenon beam of 8 keV and a neutral current of 10 µA. Melting points have been determined by Koefler hot-stage (Koefler Reichardt, Vienna, Austria) and are uncorrected.

*Synthesis of 6-*O*-acyl-1,2-isopropylidene-*D*-glucofuranoses* **4a–d**. *Method A*. A solution of **1** (0.22 g; 1.0 mmol) in dry pyridine (20 cm^3) was evaporated twice to half volume to allow azeotropic removal of traces of water. The appropriate acid chloride **2a–d** (1.25 mmol) was then added, and the mixture was stirred at room temperature for 2–6 h until a nearly complete conversion of **1** was achieved [TLC: $CHCl_3$–MeOH (90:10, vol/vol)]. Saturated aqueous $NaHCO_3$ (10 cm^3) was then added and the mixture was extracted first with ethyl ether (3 × 15 cm^3), to prevent the formation of emulsion, and then partitioned with $CHCl_3$ (3 × 15 cm^3). The chloroform layers were washed with brine and then evaporated to dryness (the pyridine present was removed by azeotropic distillation with toluene) to afford an oily residue, which was purified by short-column chromatography [$CHCl_3$–MeOH (97.5:2.5, vol/vol)] to give the products **4a–d** (Table 1).

Method B. A solution of **1** (0.22 g; 1.0 mmol) and appropriated carboxylic acid **3a–d** (1.20 mmol) in dry pyridine (20 cm^3) was evaporated twice to half volume as reported above. BOP-Cl (0.64 g; 2.5 mmol) was then added, and the mixture was stirred at room temperature for 2–24 h (Table 1). The workup and the purification of the crude final mixture were performed as for Method A.

*6-*O*-benzoyl-1,2-isopropylidene-*D*-glucofuranoses* **4a**. m.p.: 184–185°C [lit. (37): 182–184°C; 193–195°C after recrystallization]; ^1H NMR (300 MHz, [D_6]DMSO, 25°C, TMS): δ = 8.05 (*d*, 2H; $H_{2'}$, $H_{6'}$); 7.65 (*t*, 1H; $H_{4'}$); 7.55 (*t*, 2H; $H_{3'}$, $H_{5'}$); 5.85 [*d*, $^3J(H_1,H_2)$ = 3.5, 1H; H_1]; 5.35 [*d*, $^3J(H_3,OH)$ = 4.9, 1H; 3-OH]; 5.25 [*d*, $^3J(H_5,OH)$ = 5.5, 1H; 5-OH]; 4.52 [*dd*, ^2J (H_{6a},H_{6b}) = 11.1; ^3J (H_{6a}, H_5) = 1.8, 1H; H_{6a}]; 4.48, [*dd*, ^3J (H_1,H_2) = 3.5, ^3J (H_2,H_3) = 0, 1H; H_2]; 4.22 [*dd*, ^3J (H_{6b}, H_5) = 5.1, 1H; H_{6b}]; 3.97–4.15 (*m*, 3H; H_5, H_4, H_3); 1.40 (*s*, 3H; CH_3); 1.25 (*s* 3H; CH_3). MS (8 KeV, FAB, *m*-nitrobenzyl alcohol): *m/z* (%): 347 (4.7), [M + Na^+], 325

(3.8), [M + H^+], 267 (9.5), [(M − acetone)$^+$], 105 (100), [PhCO$^+$].

*6-*O*-decanoyl-1,2-isopropylidene-*D*-glucofuranoses* **4b**. m.p.: 69–70°C; 1H NMR (300 MHz, [D_6]DMSO, 25°C, TMS): δ = 5.80 (*d*, $^3J(H_1,H_2)$ = 3.6, 1H; H_1); 5.25 (*br*, 1H; 3-OH); 5.01 (*br*, 1H; 5-OH); 4.40 [*d*, $^3J(H_1,H_2)$ = 3.6, $^3J(H_2,H_3)$ = 0, 1H; H_2]; 4.20 [*dd*, $^2J(H_{6a},H_{6b})$ = 9.8, ^3J (H_{6a}, H_5) = 1.9, 1H; H_{6a}]; 4.05 [*d*, $^3J(H_3,H_4)$ = 2.5, 1H; H_3]; 3.95–3.80 (*m*, 3H; H_4, H_5, H_{6b}); 2.30 (*t*, 2H; $OCOCH_2$); 1.50 (*m*, 2H; $OCOCH_2$-CH_2); 1.40 (*s*, 3H; CH_3); 1.25 (*m*, 15H; CH_3, $(CH_2)_6$); 0.85 (*t*, 3H; CH_3). MS (8 KeV, FAB, *m*-nitrobenzyl alcohol): *m/z* (%): 397 (43), [M + Na^+], 375 (6.2), [M + H^+], 317 (50), [(M − acetone)$^+$], 155 (100), [RCO$^+$].

*6-*O*-stearoyl-1,2-isopropylidene-*D*-glucofuranoses* **4c**. m.p.: 88–89°C [lit. (7): 95°C after recrystallization]; ^{1}H NMR (300 MHz, [D_6]DMSO, 25°C, TMS): δ = 5.80 [*d*, $^3J(H_1,H_2)$ = 3.3, 1H; H_1]; 5.25 (*br*, 1H, 3-OH); 5.05 (*br*, 1H, 5-OH); 4.40 [*d*, $^3J(H_1,H_2)$ = 3.3, $^3J(H_2,H_3)$ = 0, 1H; H_2]; 4.20 (*dd*, $^2J(H_{6a},H_{6b})$ = 9.8, $^3J(H_{6a},H_5)$ = 2.5, 1H; H_{6a}]; 4.05 [*d*, $^3J(H_3,H_4)$ = 2.5, 1H; H_3]; 3.95–3.75 (*m*, 3H; H_4, H_5, H_{6b}); 2.30 (*t*, 2H; $OCOCH_2$); 1.50 (*m*, 2H; $OCOCH_2$-CH_2); 1.35 (*s*, 3H; CH_3); 1.20 (*m*, 31H; CH_3, $(CH_2)_{14}$); 0.85 (*t*, 3H; CH_3). MS (8 KeV, FAB, *m*-nitrobenzyl alcohol): *m/z* (%): 509 (100), [M + Na^+], 487 (5.5), [M + H^+], 429 (77), [(M − acetone)$^+$], 267 (74), [RCO$^+$].

*6-*O*-oleyl-1,2-isopropylidene-*D*-glucofuranoses* **4d**. m.p.: 56–57°C; ^{1}H NMR (300 MHz, [D_6]DMSO, 25°C, TMS): δ = 5.79 [*d*, $^3J(H_1,H_2)$ = 3.6, 1H; H_1]; 5.32 (*m*, 2H; CH=CH); 5.25 [*d*, $^3J(H_3,OH)$ = 3.8, 1H; 3-OH]; 5.00 (*br*, 1H, 5-OH); 4.39 [*d*, $^3J(H_1,H_2)$ = 3.6, $^3J(H_2,H_3)$ = 0, 1H; H_2]; 4.20 [*dd*, $^2J(H_{6a},H_{6b})$ = 9.8, $^3J(H_{6a},H_5)$ = 1.9, 1H; H_{6a}]; 4.05 [*d*, $^3J(H_3,H_4)$ = 2.5, 1H; H_3]; 3.95–3.85 (*m*, 3H; H_4, H_5, H_{6b}); 2.30 (*t*, 2H; $OCOCH_2$); 1.95 [*m*, 4H; CH_2-(CH=CH)-CH_2]; 1.50 (*m*, 2H; $OCOCH_2$-CH_2); 1.35 (*s*, 3H; CH_3); 1.20 [*m*, 23H; CH_3, $(CH_2)_6$, $(CH_2)_4$]; 0.85 (*t*, 3H; CH_3). MS (8 KeV, FAB, *m*-nitrobenzyl alcohol): *m/z* (%): 507 (86), [M + Na^+], 485 (4.0), [M + H^+], 427 (63), [(M − acetone)$^+$], 265 (100), [RCO$^+$].

*Synthesis of 6-*O*-acyl-α,β-*D*-glucopyranoses* **6a–d**. A solution of **4a–d** (0.50 mmol) in 70% HOAc aq. (10 cm^3) was stirred at 70°C until a nearly complete conversion of **4a–d** was achieved [TLC: $CHCl_3$–MeOH (90:10, vol/vol)]. The HOAc was removed by azeotropic distillation with toluene and the oily residue, which was purified by short-column chromatography [$CHCl_3$–MeOH (97.5:2.5, vol/vol)] to give the products **6a–d** (Table 2). The α and β isomers are clearly distinguishable by HPLC and could be separated.

*6-*O*-benzoyl-α,β-*D*-glucopyranose* **6a** *(α/β = 55:45).* ^{1}H NMR (300 MHz, [D_6]CD_3OD, 25°C, TMS): δ = 8.07 (2Hα and 2Hβ; ArH); 7.37–7.61 (3Hα and 3Hβ; ArH); 5.12 [*d*, $^3J(H_{1\alpha},H_{2\alpha})$ = 3.8, 1H; $H_{1\alpha}$]; 4.65 [*dd*, $^2J(H_{6a\beta},H_{6b\beta})$ = 11.9, $^3J(H_{6a\beta},H_5)$ = 2.1, 1H; $H_{6a\beta}$]; 4.58 [*dd*, $^2J(H_{6a\alpha},H_{6b\alpha})$ = 11.9, $^3J(H_{6a\alpha},H_5)$ = 2.3, 1H; $H_{6a\alpha}$]; 4.55 [*d*, $^3J(H_{1\beta},H_{2\beta})$ = 7.8, 1H; $H_{1\beta}$]; 4.55 [*m*, 2H, $H_{6b\alpha}$ and $H_{6b\beta}$]; 4.12 (*m*, 1H; $H_{5\alpha}$); 3.73 [*dd*, $^3J(H_2,H_3)$ = 9.3, $^3J(H_3,H_4)$ = 9.3, 1H; $H_{3\alpha}$]; 3.64 (*m*, 1H; $H_{5\beta}$); 3.37–3.48 (*m*, 4H; $H_{2\alpha}$, $H_{4\alpha}$, $H_{3\beta}$, $H_{4\beta}$); 3.20 [*dd*, $^3J(H_1,H_2)$ = 7.8, $^3J(H_2,H_3)$ = 9.0, 1H; $H_{2\beta}$]. MS (8 KeV, FAB, glycerol): *m/z* (%): 285 (5.0), [M + H^+], 267 (27), [(M − H_2O)$^+$], 105 (100), [PhCO$^+$].

*6-*O*-decanoyl-α,β-*D*-glucopyranose* **6b** *(α/β = 95:5).* ^{1}H NMR (300 MHz, [D_6]DMSO, 25°C, TMS): δ = 6.48 [*d*, $^3J(H_1,OH)$ = 6.7, 1H; 1-OH_β]; 6.34 [*d*, $^3J(H_1,OH)$ = 4.6, 1H; 1-OH_α]; 5.12 [*d*, $^3J(H_4,OH)$ = 5.4, 1H; 4-OH_β]; 5.08 [*d*, $^3J(H_4,OH)$ = 5.7, 1H; 4-OH_α]; 4.95 [*m*, 3H; 2-OH_β, 3-OH_β, $H_{1\beta}$]; 4.89 [*dd*, $^3J(H_1,H_2)$ = 4.6, $^3J(H_1,OH)$ = 4.6, 1H; $H_{1\alpha}$]; 4.78 [*d*, $^3J(H_3,OH)$ = 4.4, 1H; 3-OH_α]; 4.54 [*d*, $^3J(H_2,OH)$ = 6.7, 1H; 2-OH_α]; 4.27 [*m*, 2H; $H_{6a\alpha}$ and $H_{6a\beta}$]; 3.98 [*m*, 2H; $H_{6b\alpha}$ and $H_{6b\beta}$]; 3.76 [*m*, 1H; $H_{5\alpha}$]; 3.30 [*m*, 1H; $H_{5\beta}$]; 3.05 (*m*, 6H, $H_{2\alpha}$, $H_{3\alpha}$, $H_{4\alpha}$, $H_{2\beta}$, $H_{3\beta}$, $H_{4\beta}$); 2.28 [*m*, 2H_α and 2H_β; $OCOCH_2$]; 1.48 (*m*, 2H_α and 2H_β; $OCOCH_2$-CH_2); 1.20 [*m*, 12H_α and 12H_β; $(CH_2)_6$]; 0.85 [*m*, 3H_α and 3H_β; CH_3]. MS (8 KeV, FAB, glycerol): *m/z* (%): 335 (12), [M + H^+], 317 (96), [(M − H_2O)$^+$], 155 (100), [RCO$^+$].

*6-*O*-stearoyl-α,β-*D*-glucopyranose* **6c** *(α/β = 75:25).* ^{1}H NMR (300 MHz, [D_6]DMSO, 25°C, TMS): δ = 6.68 [*d*, $^3J(H_1,OH)$ = 6.9, 1H; 1-OH_β]; 6.36 [*d*, $^3J(H_1,OH)$ = 4.6, 1H; 1-OH_α]; 5.12 [*d*, $^3J(H_4,OH)$ = 5.4, 1H; 4-OH_β]; 5.05 [*d*, $^3J(H_4,OH)$ = 5.5, 1H; 4-OH_α]; 4.95 [*m*, 3H; $H_{1\beta}$, 2-OH_β 3-OH_β]; 4.90 [*dd*, $^3J(H_1,H_2)$ = 4.6, 1H; $H_{1\alpha}$]; 4.77 [*d*, $^3J(H_3,OH)$ = 4.7, 1H; 3-OH_α]; 4.55 [*d*, $^3J(H_2,OH)$ = 6.7, 1H; 2-OH_α)]; 4.28 [*m*, 2H; $H_{6a\alpha}$ and $H_{6a\beta}$]; 3.98 [*m*, 2H; $H_{6b\alpha}$ and $H_{6b\beta}$]; 3.78 [*m*, 1H; $H_{5\alpha}$]; 3.33 (*m*, 1H; $H_{5\beta}$); 3.07 (*m*, 6H; $H_{2\alpha}$, $H_{3\alpha}$, $H_{4\alpha}$, $H_{2\beta}$, $H_{3\beta}$, $H_{4\beta}$); 2.25 (*m*, 2H_α and 2H_β; $OCOCH_2$); 1.50 (*m*, 2H_α and 2H_β; $OCOCH_2$-CH_2); 1.21 [*m*, 28H_α and 28H_β; $(CH_2)_{14}$]; 0.85 [*m*, 3H_α and 3H_β; CH_3]. MS (8 KeV, FAB, glycerol): *m/z* (%): 437 (6), [M + H^+], 429 (32), [(M − H_2O)$^+$], 267 (100), [RCO$^+$].

*6-*O*-oleyl-α,β-*D*-glucopyranose* **6d** *(α/β = 50:50).* ^{1}H NMR (300 MHz, [D_6]DMSO, 25°C, TMS): δ = 6.69 [*d*, $^3J(H_1,OH)$ = 6.6, 1H; 1-OH_β]; 6.34 [*d*, $^3J(H_1,OH)$ = 4.6, 1H; 1-OH_α]; 5.32 (*m*, 2H_α and 2H_β; CH=CH); 5.13 [*d*, $^3J(H_4,OH)$ = 5.2, 1H; 4-OH_β]; 5.07 [*d*, $^3J(H_4,OH)$ = 5.5, 1H; 4-OH_α]; 4.96 (*m*, 3H; $H_{1\beta}$, 2-OH_β and 3-OH_β); 4.91 [*dd*, $^3J(H_1,H_2)$ = 4.6, $^3J(H_1,OH)$ = 4.6 1H; $H_{1\alpha}$]; 4.78 [*d*, $^3J(H_3,OH)$ = 4.4, 1H; 3-OH_α]; 4.55 [*d*, $^3J(H_2,OH)$ = 6.7, 1H; 2-OH_α]; 4.29 (*m*, 2H; $H_{6a\alpha}$ and $H_{6a\beta}$); 3.95 (*m*, 2H; $H_{6b\alpha}$ and $H_{6b\beta}$); 3.78 (*m*, 1H; $H_{5\alpha}$); 3.32 (*m*, 1H; $H_{5\beta}$); 3.05 (*m*, 6H; $H_{2\alpha}$, $H_{3\alpha}$, $H_{4\alpha}$, $H_{2\beta}$, $H_{3\beta}$, $H_{4\beta}$); 2.28 (*m*, 2H_α and 2H_β; $OCOCH_2$); 1.97 [*m*, 4H_α and 4H_β; CH_2-(CH=CH)-CH_2]; 1.53 (*m*, 2H_α and 2H_β; $OCOCH_2$-CH_2); 1.23 [*m*, 22H_α and 22H_β; $(CH_2)_5$, $(CH_2)_6$]; 0.85 [*m*, 3H_α and 3H_β; CH_3]. MS (8 KeV, FAB, glycerol): *m/z* (%): 437 (6), [M + H^+], 429 (32), [(M − H_2O)$^+$], 267 (100), [RCO$^+$].

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COMMUNICATION

Novel Fatty Acid Esters of (7*E*, 12*E*, 18*R*, 20*Z*)-Variabilin from the Marine Sponge *Ircinia felix*

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ABSTRACT: The methanolic extract of the marine sponge *Ircinia felix* has yielded nine novel fatty acid esters, (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin (5*Z*, 9*Z*)-22-methyltricosadienoate, (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin (5*Z*, 9*Z*)-tetracosadienoate, (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin hexadecanoate, (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 10-methylhexadecanoate, (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 15-methylhexadecanoate, (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 14-methylhexadecanoate, (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 9-octadecenoate, (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin octadecanoate, and (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 2,11-dimethyloctadecanoate, along with the recently described (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 11-methyloctadecanoate. The characterization of the new fatty acids (5*Z*, 9*Z*)-22-methyltricosadienoic and 2,11-dimethyloctadecanoic acids is also described. The chemical structures were determined by extensive spectroscopic, chromatographic, and chemical analyses.
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Marine sponges of the genus *Ircinia* (order Dictyoceratida) are known as common sources of furanosesterterpene tetronic acids and of linear C_{21} furanoterpenes (1,2). During the course of our studies on *I. felix*, *I. campana,* and *I. strobilina* occurring in the Colombian Caribbean coast, we recently reported (3,4) on the occurrence of (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin as the major antimicrobial furanosesterterpene tetronic acid in the three *Ircinia* sponges examined, followed by medium levels of (8*E*, 13*Z*, 18*R*, 20*Z*)-strobilinin, (7*E*, 13*Z*, 18*R*, 20*Z*)-felixinin and lesser amounts of (8*Z*, 13*Z*, 18*R*, 20*Z*)-strobilinin and (7*Z*, 13*Z*, 18*R*, 20*Z*)-felixinin. More recently we detected in *I. felix* a complex mixture of fatty acid esters of furanosesterterpene tetronic acids, of which only the novel variabilin 11-methyloctadecanoate has so far been reported (5).

To continue our studies on *I. felix* collected in the Colombian Caribbean, we reisolated the furanosesterterpene tetronic acid fatty acid ester mixture in order to identify the rest of the esters present in this marine sponge. Thus, in this paper we report on the isolation of nine novel fatty acid esters of (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin as major components of the above-mentioned mixture along with two new fatty acids, whose chemical structures were determined by spectroscopic, chromatographic, and chemical analyses.

MATERIALS AND METHODS

Materials. All solvents used were of high purity at purchase (Merck, Darmstadt, Germany) and were redistilled before use.

Samples. Ircinia felix was collected by SCUBA at Santa Marta Bay on the Caribbean coast of Colombia, at a depth of 15–20 m. Voucher specimens have been deposited in the reference collection of the Instituto de Investigaciones Marinas de Punta Betín, Colombia. Local populations have been described by Zea (6).

Variabilin fatty acid esters extraction. A sample of thawed sponge (1.8 kg) was soaked in methanol (2 L) for 24 h and filtered. After concentration of the extract, it was partitioned between ethyl acetate/water (2:1, vol/vol). The organic layer was dried, and removal of the solvent yielded a brown residue (19.4 g). Part of this residue (4.4 g) was subjected to column chromatography on silica gel, eluting with hexane/ethyl acetate (5:1, vol/vol) to give 19 mg of crude variabilin fatty acid ester mixture. Although this fraction was detected as a single spot by thin-layer chromatography (TLC) analysis on silica gel, reversed-phase high-performance liquid chromatography (RP-HPLC) revealed it to be a complex mixture of esters which were separated on a preparative ODS HPLC column and identified on the basis of spectral, chromatographic, and chemical evidence.

High-performance liquid chromatography (HPLC). Preparative HPLC was performed on a Shimadzu Techno-Research PREP-ODS(H) column (250 × 20 mm i.d., 5 μm) using a Shimadzu Liquid Chromatograph 6A (Kyoto, Japan) and a Shimadzu SDD-6A ultraviolet (UV)-visible detector monitoring at 270 nm, with methanol as mobile phase (flow rate 5 mL/min). Fractions were collected as indicated by dotted lines in Figure 1.

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Abbreviations: ECL, equivalent chain length; EI–MS, electron impact mass spectrometry; FAB–MS, fast atom bombardment–MS; GC, gas chromatography; GC/MS, GC coupled to MS; HPLC, high-performance liquid chromatography; NBA, nitrobenzylalcohol; NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high-performance liquid chromatography; RT, retention time; TLC, thin-layer chromatography; UV, ultraviolet.

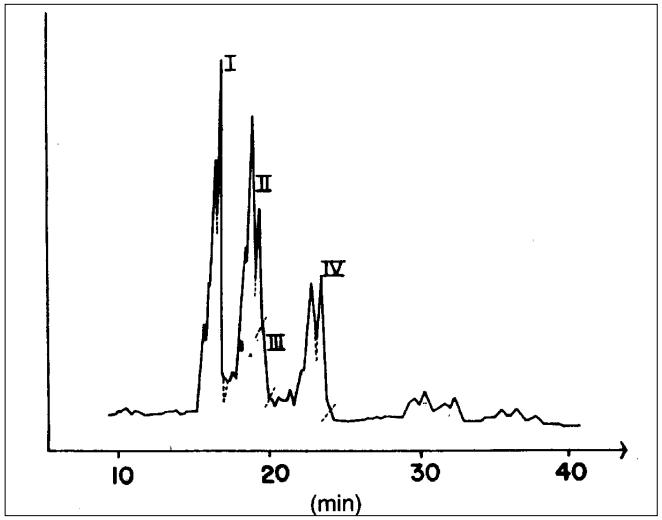


FIG. 1. High-performance liquid chromatography of the furanosesterterpene tetronic acid fatty acid ester mixture isolated from the marine sponge *Ircinia felix* on a PREP-ODS(H) column (250 × 20 mm i.d., 5 μm) (Shimadzu, Kyoto, Japan). Elution conditions are given in the Materials and Methods section. Fractions I–IV were as indicated by the dotted line, collected by monitoring at 270 nm.

Spectroscopic analyses of variabilin fatty acid esters. Fatty acid furanosesterterpene tetronic acids isolated by HPLC were subjected to analyses by absorption UV, electron impact–mass spectrometry (EI–MS) and/or fast atom bombardment–MS (FAB–MS) and ^{1}H nuclear magnetic resonance (NMR). UV spectra of methanol solutions were taken on a Beckman 25 instrument (Fullerton, CA). The mass spectra were determined with a Jeol AX505HA (Tokyo, Japan) instrument in the electron impact and/or in the FAB mode. In the former case 70 eV as electron energy was employed, maintaining the ion source at 305 C. A matrix of 3-nitrobenzyl alcohol (NBA), emission current of 10 mA, and an accelerating voltage of 6 KeV were used during MS in the FAB mode. ^{1}H NMR spectra ($CDCl_3$) were obtained using a Jeol JNM-EX400 instrument at 400 MHz. Chemical shifts are given in ppm relative to tetramethylsilane as internal standard.

Acid hydrolysis of the separated variabilin fatty acid esters. Each of the fatty acid ester fractions (about 1 mg each) isolated by HPLC was treated with 5 mL of 2 N HCl in methanol for 2 h. The hydrolysis products (furanosesterterpene tetronic acids and fatty acids) were extracted with diethyl ether. Half of this extract was derivatized with CH_2N_2 to afford the fatty acid methyl ester and the furanosesterterpene tetronic acid methyl ether derivatives which were characterized by capillary gas chromatography (GC) and capillary GC/MS analyses. Subsequently one part of the fatty acid methyl ester derivatives was converted into *N*-acyl pyrrolidides (7), and the other part was tranformed into *p*-bromophenacyl ester derivatives (obtained by treating the fatty acid methyl esters with *p*-bromophenacylbromide and diisopropylethylamine in methanol, followed by HPLC purification). The pyrrolidide derivatives were analyzed by capillary GC and capillary GC/MS and the *p*-bromophenacyl esters by ^{1}H NMR under the same conditions and instruments described above for variabilin fatty acid esters. The other part of the hydrolysate was treated with acetic anhydride/pyridine (1:1, vol/vol) giving variabilin acetate which was characterized by direct comparison on HPLC with an authentic sample of (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin acetate.

Capillary GC analyses. The fatty acid methyl esters and the variabilin methyl ether were analyzed by GC using a Hewlett-Packard 5890 (Palo Alto, CA) gas chromatograph series II with flame-ionization detector (FID) equipped with a Hewlett-Packard crosslinked OV-101 fused silica capillary column (25 m × 0.25 mm i.d.; film thickness, 0.25 μm). The temperature program was started at 170°C and raised at 2°C/min to 270°C. Flow rates of carrier and make-up gases were 1 mL/min and 30 mL/min, respectively. Detector and injector temperatures were kept at 250 and 300°C, respectively. The *N*-acyl pyrrolidides were analyzed under the same conditions used for the methyl esters except that the column temperature program was started at 230°C and raised at 4°C/min to 290°C.

Capillary GC/MS analyses. The GC/MS analyses were carried out on a Hewlett-Packard 5970 mass selective detector directly coupled to a Hewlett-Packard 5890 gas chromatograph series II, using the same conditions as mentioned above for capillary GC analyses of methyl ester and pyrrolidide derivatives. Other MS conditions were as follows: electron energy 70 eV and mass range 30–500. Below are the complete spectral data for the new esters.

Fraction I [(7*E*, 12*E*, 18*R*, 20*Z*)-Variabilin 22-methyl-5,9-tricosadienoate, **1,** + (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 5,9-tetracosadienoate, **2**)]. Colorless oil; UV and ^{1}H NMR data, *cf.* the Results and Discussion section. Acid hydrolysis of fraction I yielded 22-methyl-5,9-tricosadienoic acid, 5,9-tetracosadienoic acid and (7*E*, 12*E*, 20*Z*)-variabilin whose spectral data in their derivatized form are as follows: 22-methyl-5,9-tricosadienoic acid methyl ester: equivalent chain length (ECL) = 22.94; MS *m/z* (relative intensity) 378 (M^+, 19), 347 (9), 346 (10), 329 (4), 318 (1), 304 (8), 281 (22), 250 (7), 236 (11), 208 (15), 207 (27), 191 (9), 181 (11), 167 (10), 164 (10), 150 (23), 141 (44), 136 (22), 121 (17), 109 (45), 96 (33), 95 (35), 85 (12), 81 (100), 74 (21), 71 (23), 69 (51), 68 (34), 67 (76), 57 (42), 55 (80). *N*-(22-methyl-5,9-tricosadienoyl)-pyrrolidide: MS *m/z* (relative intensity) 417 (M^+, 2), 234 (5), 220 (1), 206 (1), 194 (1), 180 (16), 166 (1), 152 (1), 140 (1), 126 (13), 113 (100), 98 (25), 84 (10), 81 (5), 72 (25), 70 (20), 55 (50), 43 (70), 41 (40). 22-Methyl-5,9-tricosadienoic acid *p*-bromophenacyl ester: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86 (6H, *d*, *J* = 6.8 Hz, 22-Me, 23-Me), 1.26 (intense *br s*), 1.70 (2H, *qui*, *J* = 6.8 Hz, 3-H), 2.02 (2H, *q*, *J* = 6.8 Hz, 11-H), 2.08 (4H, *m*, 7-H, 8-H), 2.13 (2H, *q*, *J* = 6.8 Hz, 4-H), 2.48 (2H, *t*, *J* = 6.8 Hz, 2-H), 5.29 (2H, *s*), 5.32–5.46 (4H, *m*, 5-H, 6-H, 9-H, 10-H), 7.64 (2H, *d*, *J* = 7.8 Hz), 7.79 (2H, *d*, *J* = 7.8 Hz). ECL and MS data of 5,9-tetracosadienoic acid methyl ester and pyrrolidide were in good agreement with those previously reported (8,9). 5,9-Tetracosadienoic acid *p*-bromophenacyl ester: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (3H, *t*, *J* = 6.8 Hz, 24-Me), 1.26 (intense *br s*), 1.70 (2H, *qui*, *J* = 6.8

Hz, 3-H), 2.02 (2H, *q*, *J* = 6.8 Hz, 11-H), 2.08 (4H, *m*, 7-H, 8-H), 2.13 (2H, *q*, *J* = 6.8 Hz, 4-H), 2.49 (2H, *t*, *J* = 6.8 Hz, 2-H), 5.29 (2H, *s*), 5.32–5.46 (4H, *m*, 5-H, 6-H, 9-H, 10-H), 7.64 (2H, *d*, *J* = 7.8 Hz), 7.79 (2H, *d*, *J* = 7.8 Hz). (7*E*, 12*E*, 20*Z*)-Variabilin methyl ether: retention time (RT) in capillary GC = 35.3 min; MS *m/z* (relative intensity) 412 (M^+, 8), 397 (5), 331 (2), 203 (5), 167 (44), 149 (20), 135 (45), 123 (30), 109 (14), 95 (15), 81 (100), 69 (30), 55 (28). (7*E*, 12*E*, 20*Z*)-Variabilin acetate: RT in HPLC = 11.8 min.

Fraction II [(7*E*, 12*E*, 18*R*, 20*Z*)-variabilin hexadecanoate, **3**, + (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 10-methylhexadecanoate, **4**)]. Colorless oil. UV (λ_{max} 275 nm); FAB–MS data, the Results and Discussion section; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84 (*d*, *J* = 6.8 Hz), 0.88 (*t*, *J* = 6.4 Hz), 1.05 (*d*, *J* = 6.4 Hz), 1.26 (intense *br s*), 1.55 (*s*), 1.58 (*s*), 1.70 (*qui*, *J* = 6.8 Hz), 1.81 (*s*), 2.60 (*t*, *J* = 6.4 Hz), 2.82 (*m*), 5.04 (*d*, *J* = 9.8 Hz), 5.08 (*t*, *J* = 6.8 Hz), 5.17 (*t*, *J* = 6.8 Hz), 6.28 (*s*), 7.20 (*s*), 7.34 (*s*). Hydrolysis of fraction II yielded hexadecanoic acid, 10-methylhexadecanoic acid, and (7*E*, 12*E*, 20*Z*)-variabilin. ECL and MS data of hexadecanoic acid derivatives (methyl ester and pyrrolidide) were the same as reported by Duque *et al.* (8). Hexadecanoic acid *p*-bromophenacyl ester: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (3H, *t*, *J* = 6.8 Hz, 16-Me), 1.26 (intense *br s*), 1.70 (2H, *qui*, *J* = 6.8 Hz, 3-H), 2.48 (2H, *t*, *J* = 6.8 Hz, 2-H), 5.29 (2H, *s*), 7.64 (2H, *d*, *J* = 7.8 Hz), 7.79 (2H, *d*, *J* = 7.8 Hz). ECL and MS data of 10-methylhexadecanoic acid methyl ester and pyrrolidide are in good agreement with those reported previously by us (8). 10-Methylhexadecanoic acid *p*-bromophenacyl ester: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.83 (3H, *d*, *J* = 6.8 Hz, 10-Me), 0.88 (3H, *t*, *J* = 6.8 Hz, 16-Me), 1.26 (intense *br s*), 1.70 (2H, qui, *J* = 6.8 Hz, 3-H), 2.49 (2H, *t*, *J* = 6.8 Hz, 2-H), 5.29 (2H, *s*), 7.64 (2H, *d*, *J* = 7.8 Hz), 7.79 (2H, *d*, *J* = 7.8 Hz). GC, GC/MS and HPLC data of (7*E*, 12*E*, 20*Z*)-variabilin methyl ether and acetate derivatives were found to be the same as for fraction I.

Fraction III [(7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 15-methylhexadecanoate, **5**, + (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 14-methylhexadecanoate, **6**)]. Colorless oil. UV (λ_{max} 275 nm); EI–MS spectrum showed only the characteristic fragments of the (7*E*, 12*E*, 20*Z*)-variabilin moiety (3); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86 (*d*, *J* = 6.4 Hz), 0.88 (*t*, *J* = 6.4 Hz), 1.05 (*d*, *J* = 6.8 Hz), 1.26 (intense *br s*), 1.55 (*s*), 1.58 (*s*), 1.70 (*qui*, *J* = 6.8 Hz), 1.81 (*s*), 2.60 (*t*, *J* = 6.4 Hz), 2.82 (*m*), 5.04 (*d*, *J* = 9.8 Hz), 5.08 (*t*, *J* = 6.8 Hz), 5.17 (*t*, *J* = 6.8 Hz), 6.28 (*s*), 7.20 (*s*), 7.34 (*s*). Hydrolysis of fraction III yielded 15-methylhexadecanoic acid, 14-methylhexadecanoic acid, and (7*E*, 12*E*, 20*Z*)-variabilin. ECL and GC/MS data of methyl ester and pyrrolidide derivatives of 15-methylhexadecanoic and of 14-methylhexadecanoic were the same as reported by Duque *et al.* (8). 15-Methylhexadecanoic acid *p*-bromophenacyl ester: ^{1}H NMR (400 MHZ, $CDCl_3$) δ 0.86 (6H, *d*, *J* = 6.8 Hz, 15-Me, 16-Me), 1.26 (intense *br s*), 1.70 (2H, *qui*, *J* = 6.8 Hz, 3-H), 2.48 (2H, *t*, *J* = 6.8 Hz, 2-H), 5.29 (2H, *s*), 7.64 (2H, *d*, *J* = 7.8 Hz), 7.79 (2H, *d*, *J* = 7.8 Hz). GC, GC/MS, and HPLC data of (7*E*, 12*E*, 20*Z*)-variabilin methyl ether and acetate derivatives were the same as reported for fraction I.

Fraction IV [(7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 9-octadecenoate, **7**, + (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin octadecanoate, **8**, + (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 11-methyloctadecanoate, **9**, + (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 2,11-dimethyloctadecanoate, **10**)]. Colorless oil. UV (λ_{max} 275 nm); FAB–MS *m/z* (relative intensity) 693 ($[M + H]^+$, 1), 679 ($[M + H]^+$, 5), 665 ($[M + H]^+$, 1), 663 ($[M + H]^+$, 1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84 (*d*, *J* = 6.8 Hz), 0.88 (*t*, *J* = 6.4 Hz), 1.05 (*d*, *J* = 6.4 Hz), 1.26 (intense *br s*), 1.55 (*s*), 1.58 (*s*), 1.70 (*qui*, *J* = 6.8 Hz), 1.81 (*s*), 2.60 (*t*, *J* = 6.4 Hz), 2.82 (*m*), 5.04 (*d*, *J* = 10.8 Hz), 5.08 (*t*, *J* = 6.8 Hz), 5.17 (*t*, *J* = 6.8 Hz), 6.28 (*s*), 7.20 (*s*), 7.34 (*s*). Hydrolysis of fraction IV afforded 9-octadecenoic acid, octadecanoic acid, 11-methyloctadecanoic acid, 2,11-dimethyloctadecanoic acid, and (7*E*, 12*E*, 20*Z*)-variabilin. ECL and GC/MS data of 9-octadecenoic acid and octadecanoic acid derivatives (methyl esters and pyrrolidides) were in good agreement with those reported (8–10). ECL and GC/MS data of 11-methyloctadecanoic acid (methyl ester and pyrrolidide derivatives) and ^{1}H NMR data of 11-methyloctadecanoic acid *p*-bromophenacyl ester were the same as reported by Martínez *et al.* (5). 2,11-Dimethyloctadecanoic acid methyl ester: ECL = 19.14, MS *m/z* (relative intensity) 326 (M^+, 25), 297 (1), 283 (19), 269 (3), 255 (2), 241 (4), 227 (17), 213 (4), 199 (20), 185 (5), 171 (8), 157 (40), 143 (14), 129 (6), 115 (17), 101 (83), 88 (100), 85 (22), 83 (24), 71 (28), 69 (34), 57 (39), 55 (50). GC, GC/MS and HPLC data of (7*E*, 12*E*, 20*Z*)-variabilin methyl ether and acetate derivatives were the same as reported for fraction I.

RESULTS AND DISCUSSION

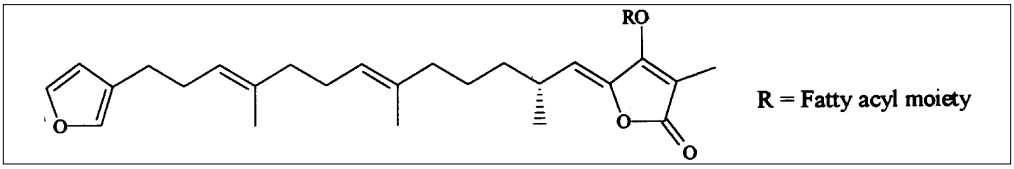
As seen in Table 1, HPLC analysis of the fatty acid ester fraction obtained from the marine sponge *I. felix* showed it to be a highly complex mixture. Previously we have separated only one compound, (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin 11-methyloctadecanoate, **9**, from this mixture (5). However, further investigation on the same ester mixture has resulted in the isolation and identification of nine new esters (compounds **1**–**8** and **10**). Compounds **1**–**10** (Table 1) constituted about 49.5% of the ester mixture. The esters and their hydrolyzed products were readily identified in fractions I–IV (Fig. 1) by chemical, chromatographic, and spectroscopic methods as described below.

Fraction I. FAB–MS spectrum of fraction I could not be obtained. However, UV (λ_{max} 275 nm) analysis and ^{1}H NMR spectrum showing the characteristic signals of (7*E*, 12*E*, 20*Z*)-variabilin acetate (3), e.g., δ 1.05 (*d*, *J* = 6.6 Hz), 1.55 (*s*), 1.58 (*s*), 1.82 (*s*), 2.82 (*m*), 5.04 (*d*, *J* = 10.8 Hz), 5.08 (*t*, *J* = 6.8 Hz), 5.17 (*t*, *J* = 6.8 Hz), 6.28 (*s*), 7.20 (*s*), and 7.33 (*s*), and additional signals for one primary and two secondary methyl groups, a triplet at δ 2.60 ppm (*J* = 6.8 Hz) corresponding to an α-methylene to a carbonyl group, a quintet at δ 1.70 ppm assignable to a β-methylene to a carbonyl, and a multiplet at δ 5.35 ppm due to the resonance of olefinic protons clearly indicated the presence of at least two variabilin fatty acid esters (compounds **1** and **2**). Hydrolysis of fraction I followed by treatment with CH_2N_2 afforded 22-methyl-5,9-

TABLE 1.
Fatty Acid Esters of (7*E*, 12*E*, 18*R*, 20*Z*)-Variabilin from the Marine Sponge *Ircinia felix*

Compound number	Fatty acid linked to (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin[a]	Molecular weight	Abundance[b] (%)
1	(5*Z*, 9*Z*)-22-Methyltricosadienoic[c]	744	7.2
2	(5*Z*, 9*Z*)-Tetracosadienoic	744	13.7
3	*n*-Hexadecanoic	636	7.9
4	10-Methylhexadecanoic	650	8.3
5	15-Methylhexadecanoic	650	0.7
6	14-Methylhexadecanoic	650	1.0
7	9-Octadecenoic	662	0.7
8	*n*-Octadecanoic	664	0.5
9	11-Methyloctadecanoic	678	7.3
10	2,11-Dimethyloctadecanoic[c]	692	2.2

[a]



[b]In total isolated esters.
[c]Fatty acids reported here by the first time in nature.

tricosadienoic (M^+ 378, ECL = 22.94) and 5,9-tetracosadienoic (M^+ 378, ECL = 23.56) (8) methyl esters (characterized by capillary GC and capillary GC/MS analyses), and (7*E*, 12*E*, 20*Z*)-variabilin methyl ether which was also identified by capillary GC and GC/MS. Evidence for the location of the double bonds was obtained from the intense fragments at *m/z* 126 and 234 (allylic cleavages of the 3-4 and 11-12 bonds) and at *m/z* 180 (bisallylic cleavage of the 7-8 bond) in the pyrrolidide derivative mass spectra of 22-methyl-5,9-tricosadienoic and 5,9-tetracosadienoic acids. Double-bond unsaturations at C-5 and C-9 were also recognized in the pyrrolidide mass spectra of both derivatives due to a separation of 12 amu between fragments at *m/z* 140 and 152, and at *m/z* 194 and 206. It is known from the mass spectra of fatty acid pyrrolidides that if an interval of 12 amu, instead of the regular 14, is observed between the most intense peaks of clusters of fragments containing *n* and $n - 1$ carbon atoms in the acid moiety, then a double bond was present between carbon *n* and $n + 1$ in the molecule (7). The final evidence for the fatty acid structures was given by the ^{1}H NMR spectra of their *p*-bromophenacyl ester derivatives. The *p*-bromophenacyl ester of acid derived from **1** showed a signal at δ 0.86 ppm (6H, *d*, *J* = 6.8 Hz) due to 22- and 23-methyl groups in the ^{1}H NMR, whereas the same ester derived from **2** exhibited a signal at δ 0.88 ppm (3H, *t*, *J* = 6.8 Hz) due to a terminal methyl group. The geometry of the olefinic bonds was deduced from ^{1}H NMR spectral comparison of the two *p*-bromophenacyl esters (400 MHz) with methyl 22-methyl-5*Z*, 9*Z*-octacosadienoate (9). The chemical shifts (δ 5.32–5.46 ppm) as well as the shapes of the olefinic protons (H-5, -6, -9, and -10) of the *p*-bromophenacyl esters were essentially identical with those of the reference spectrum. Further, the chemical shifts and shapes of the allylic methylene protons (δ 2.08 ppm for H-7 and H-8, and δ 2.02 for H-11) were also closely similar to those of the reference spectrum. The methylene protons ascribable to C-2, C-3, and C-4 resonated at slightly shifted positions compared to the respective protons in the reference spectrum because of the difference in the ester group. These data are in support of a 5*Z*, 9*Z*-olefinic structure for the fatty acid portion of **1** and **2**. Additionally the alcoholic portion of the two esters of fraction I was also confirmed by treatment of the other part of the hydrolysate with Ac_2O-pyridine to afford (7*E*, 12*E*, 20*Z*)-variabilin acetate which was characterized as such by direct comparison on HPLC with an authentic sample. Based on these analyses, the structures of compounds **1** and **2** were established as (7*E*, 12*E*, 20*Z*)-variabilin (5*Z*, 9*Z*)-22-methyltricosadienoate and (7*E*, 12*E*, 20*Z*)-variabilin (5*Z*, 9*Z*)-tetracosadienoate, respectively.

Fraction II. FAB–MS spectrum of the separated fraction II exhibited two pseudomolecular ions $(M + H)^+$ at *m/z*: 637 and 651, indicating the presence of two furanosesterterpene esters (compounds **3** and **4**) of molecular formulas $C_{41}H_{64}O_5$ (the acid portion being a saturated C_{16}-acid) and $C_{42}H_{66}O_5$ (the acid portion being a saturated C_{17}-acid), respectively. The UV and ^{1}H NMR spectra clearly indicated the structure of (7*E*, 12*E*, 20*Z*)-variabilin for the alcoholic part and saturated fatty acids for the acyl moieties of compounds **3** and **4**. Alkaline hydrolysis followed by derivatization (methyl esters, pyrrolidides, and *p*-bromophenacylesters for the fatty acids and methyl ether and acetate for the furanosesterterpenyl moiety) afforded *n*-hexadecanoic and 10-methylhexadecanoic acids which were characterized by capillary GC and capillary GC/MS (8) and ^{1}H NMR analyses, and (7*E*, 12*E*, 20*Z*)-variabilin identified by capillary GC, GC/MS, and by direct comparison with an authentic sample in HPLC. These analyses clearly supported the structure of compound **3** as (7*E*, 12*E*, 20*Z*)-variabilin *n*-hexadecanoate and of compound **4** as (7*E*, 12*E*, 20*Z*)-variabilin 10-methylhexadecanoate.

Fractions III and IV. Fraction III was found to be composed of compounds **5** and **6** using the same methods as described above, and their structures were determined as (7*E*, 12*E*, 20*Z*)-variabilin 15-methylhexadecanoate and (7*E*, 12*E*, 20*Z*)-variabilin 14-methylhexadecanoate, respectively. Finally, investigation of fraction IV showed it to be a mixture of (7*E*, 12*E*, 20*Z*)-variabilin 9-octadecenoate, **7**; (7*E*, 12*E*, 20*Z*)-variabilin *n*-octadecanoate, **8**; (7*E*, 12*E*, 20*Z*)-variabilin 11-methyloctadecanoate, **9**; and (7*E*, 12*E*, 20*Z*)-variabilin 2,11-dimethyloctadecanoate, **10**. The 2,11-dimethyloctadecanoic acid was readily characterized by the analysis of its mass spectrum as methyl ester derivative. The molecular ion at *m/z* 326 was consistent with the molecular formula $C_{21}H_{42}O_2$, the fragment ion at *m/z* 88 indicated an acyl moiety having methyl substitution at C-2, and the intense fragments *m/z* 227 and 199, simultaneously with the diminished C_{11} fragment *m/z* 213, clearly showed 11-methyl branching.

The C-18 configuration for the ten (7*E*, 12*E*, 20*Z*)-variabilin fatty acid esters isolated from *I. felix* could be reasonably assigned as *R* based on the fact that this species contains enantiomerically pure (18*R*)-(7*E*, 12*E*, 20*Z*)-variabilin as the free form, and hydrolysis of a crude variabilin fatty acid mixture followed by acetylation and HPLC separation afforded (+)-(18*R*)-variabilin acetate (3).

We have recently characterized five furanosesterterpene tetronic acids, (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin, (7*E*, 13*Z*, 18*R*, 20*Z*)-felixinin, (8*E*, 13*Z*, 18*R*, 20*Z*)-strobilinin, (7*Z*, 13*Z*, 18*R*, 20*Z*)-felixinin, and (8*Z*, 13*Z*, 18*R*, 20*Z*)-strobilinin in the methanolic extract of *I. felix* (4). It is therefore reasonable to assume that the other four furanosesterterpenes occur in fatty acid bound form as well. Indeed, these furanosesterterpenes were identified in the hydrolysate of the total ester mixture in the present study (data not shown). A variety of combinations of the five furanosesterterpenes and 10 (or more) fatty acids could account for the complex HPLC pattern (Fig. 1).

Linear sesterterpenes characterized by a furan ring at one end and by a conjugated tetronic acid at the other, e.g., (7*E*, 12*E*, 20*Z*)-variabilin, are common natural products in marine sponges of the genus *Ircinia*. Similarly, most of the fatty acids [except (5*Z*, 9*Z*)-22-methyltricosadienoic acid and 2,11-dimethyloctadecanoate] bound to (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin had already been detected as phospholipid components in *Ircinia* species (11) including *I. felix* collected from other Caribbean sites. However, the rare combination of fatty acids linked to (7*E*, 12*E*, 18*R*, 20*Z*)-variabilin has no precedent in nature, and, this paper reports for the first time the presence in nature of the (5*Z*, 9*Z*)-22-methyltricosadienoic and 2,11-dimethyloctadecanoic acids.

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ARTICLE

Expression of Fatty Acyl-CoA Binding Proteins in Colon Cells: Response to Butyrate and Transformation

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ABSTRACT: Fatty acyl-CoA affect many cellular functions as well as serving as cellular building blocks. Several families of cytosolic fatty acyl-CoA binding proteins may modulate the activities of fatty acyl-CoA. Intestinal enterocytes contain at least three unique families of cytosolic proteins that bind fatty acyl-CoA: acyl-CoA binding protein (ACBP), fatty acid binding proteins (including the liver, L-FABP and intestinal, I-FABP), and sterol carrier protein-2 (SCP-2). Immortalized rat colon epithelial cell lines expressed only ACBP and SCP-2 at levels of 0.75 ± 0.13 and 0.42 ± 0.02 ng/μg protein. *Ras* and *src* transformation increased colon cell density and differentially altered ACBP and SCP-2 expression without affecting I-FABP or L-FABP levels. ACBP levels were 1.8-fold and 1.5-fold increased in *ras*- and *src*-transformed cells, respectively. In contrast, SCP-2 expression was significantly decreased 55 and 67% in *ras*- and *src*-transformed cells, respectively. Butyrate treatment of *ras*- and *src*-transformed cells decreased cell proliferation up to 60–85% as compared to 25–30% in control cells. Butyrate treatment decreased ACBP expression in all cell lines but had no effect on the levels of SCP-2, I-FABP, or L-FABP. These studies suggest that the differential expression of ACBP and SCP-2 in rat colonic cell lines, as well as their modulation by butyrate, may be altered by cell transformation.

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Colorectal adenocarcinoma is one of the leading causes of death due to cancer in North America (1). A number of environmental and genetic factors have been associated with the development and progression of colon carcinoma. Genetic changes include loss of potential tumor suppressor genes as well as activation of oncogenes (2,3). Activation of K-*ras* by a point mutation occurs in >40–50% of colon carcinomas (3). Activation of the tyrosine kinase of the c-*src* gene product ($pp60^{c\text{-}src}$) is also common, and activation occurs early in tumor development for both of these oncogenes (2,3). Several animal models and *in vitro* systems have been developed for analysis of factors contributing to tumor progression. Due to the inability to maintain long-term cultures of normal colon epithelial cells *in vitro,* immortalized, nontumorigenic fetal rat colon epithelial cell lines have been developed as a model and several oncogenes introduced to produce transformed, neoplastic cell lines (4). This model is useful for examination of the effects of transformation on the expression of various proteins within the cells as well as effects of therapeutic agents on colon cell proliferation.

Butyrate is a volatile short-chain fatty acid which is a by-product of bacterial degradation of certain types of dietary fiber (5). It has growth inhibitory and differentiation effects on various cell types, including colon cells, *in vitro* (reviewed 6,7). Butyrate affects cell proliferation by inhibiting DNA synthesis and arresting cells in G1 phase of the cell cycle (7). *In vivo,* butyrate has trophic effects on normal colonic epithelium and may inhibit growth of neoplastic cells (reviewed 6,7). In one study using a rat model of experimental colon epithelial cell neoplasia, feeding of fiber associated with production of high levels of butyrate correlated with lower tumor mass (5). In addition, butyrate administered by intravenous infusion inhibited growth of malignant colon carcinoma cells metastatic to the liver in mice (7).

Acyl-CoA binding protein (ACBP), fatty acid binding proteins (FABP), and sterol carrier protein-2 (SCP-2) represent three families of low molecular weight, cytosolic proteins that bind fatty acyl-CoA, and *via* this interaction may have an effect on cell signaling pathways involved in cell proliferation (reviewed 8). ACBP is a 10 kDa protein that was first discovered in 1983 in the brain and designated diazepam binding inhibitor based upon its ability to modulate $GABA_A$ (γ-amino butyric acid) receptors (9). In 1987, the same protein, designated acyl CoA-binding protein, was isolated from bovine liver and found to induce the synthesis of medium-chain acyl-CoA (10). ACBP is highly conserved from yeasts to mammals and has been found in all mammalian tissues tested (reviewed 8). It is abundant in normal liver and intestinal tract, including the colon. Levels almost as high as seen in liver (0.15 nmol/g wet weight) have been determined in rabbit colon (0.11 nmol/g wet weight) (reviewed 8).

FABP such as liver-FABP (L-FABP) and intestinal-FABP

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Abbreviations: ACBP, acyl-CoA binding protein; DMEM, Dulbecco's Minimal Essential Medium; FABP, fatty acid binding proteins; $GABA_A$, γ-amino butyric acid; I-FABP, intestinal fatty acid binding protein; L-FABP, liver fatty acid binding protein; PBR, peripheral benzodiazepine receptor; PBS, phosphate buffered saline; PMSF, phenylmethyl sulfonyl fluoride; PVDF, polyvinylidene fluoride; SCP-2, sterol carrier protein-2; TBS, tris buffered saline; TBST, tris buffered saline + 0.05% Tween 20.

(I-FABP) belong to a large group of proteins including over 17 distinct members (11). These proteins are normally present in high concentration within the cell cytoplasm and bind hydrophobic ligands such as fatty acids, prostaglandins, and fatty acyl-CoA (12). L-FABP is expressed in high abundance in normal liver and small intestine but in reduced levels in the colon (13). I-FABP, which binds fatty acids (14) and fatty acyl-CoA (8), is expressed only in the intestinal tract but also exhibits a gradient of expression so that low levels are found in normal stomach and colon (13). In addition, SCP-2, also known as nonspecific lipid transfer protein, binds such ligands as fatty acids, phospholipids, glycolipids, cholesterol, and fatty acyl-CoA (8,14–16). Two forms of the protein are detectable by immunostaining. The 58 kDa form is present only in peroxisomes, while the 15 and 13.2 kDa forms of the protein are present in cytosol, mitochondria, peroxisomes, and near the endoplasmic reticulum (17). The 15 kDa pro-SCP-2 is not often detected in tissues because it undergoes rapid posttranslational cleavage of an N-terminal leader fragment to yield the mature 13.2 kDa form (18). SCP-2 protein levels are highest in normal rat liver and intestinal mucosal epithelial cells, with lower levels in other tissues (17).

Of these four fatty acyl-CoA binding proteins, only L-FABP and I-FABP expression have been examined in colon adenomas and adenocarcinomas. Carroll *et al*. (19) showed decreased expression of L-FABP in human colorectal adenomas and adenocarcinomas by Western immunoblotting and a mosaic of L-FABP positive cells by immunohistochemistry. In another study with experimental rat colon adenocarcinoma, a 10-fold and 50-fold decrease in I-FABP and L-FABP mRNA, respectively, was observed when compared with normal colon mucosal epithelial cells (20). Immunohistochemical analysis confirmed the decreased expression of I-FABP and L-FABP protein and showed that expression occurred in isolated foci of epithelial cells. Results of these studies suggest that changes in FABP expression may occur early in tumor development (adenomas exhibited the same pattern of immunostaining as carcinomas) and that FABP expression depends upon differentiation and possibly proliferative capacity of the cells. The purpose of the present study was to examine the effects of transformation with *ras* and *src* oncogenes and the effects of butyrate treatment on the expression of the four fatty acyl-CoA binding proteins—ACBP, L-FABP, I-FABP, and SCP-2—on colon epithelial cells grown *in vitro*.

MATERIALS AND METHODS

Materials. Prestained low molecular weight protein standards were purchased from Gibco (Gaithersburg, MD). Phenylmethyl sulfonyl fluoride (PMSF), leupeptin, pepstatin-A, EDTA, Trizma base, SDS, 4-chloro-1-naphthol, acrylamide, *n,n*-methylene bis-acrylamide, bovine actin, mouse anti-actin monoclonal antibody, 85% phosphoric acid, Coomassie brilliant blue G250, glycerol, bovine serum albumin, William's Medium E, Dulbecco's Minimal Essential Medium (DMEM), insulin, hydrocortisone, and holo-transferrin were obtained from Sigma Chemical Company (St. Louis, MO). Glycine, Tween 20, Tricine, 2-mercaptoethanol, methanol, ethanol, polyvinylidene fluoride (PVDF), and all sterile, disposable, tissue culture plasticware were from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum was from Hyclone Labs (Logan, UT). Horseradish peroxidase conjugated goat antirabbit IgG was purchased from Bio-Rad (Hercules, CA). Biotinylated goat antirabbit IgG, biotinylated goat antimouse IgG, ABC alkaline phosphatase kit, and Vector black substrate kit were from Vector (Burlingame, CA). Rat liver ACBP and recombinant I-FABP, L-FABP, and SCP-2 were purified as previously described (21–23). L-[4,5-^{3}H] leucine (52 Ci/mmol) was from NEN Products (Boston, MA).

Antibody production. Polyclonal rabbit antirat liver ACBP, rabbit antirecombinant I-FABP, rabbit antirecombinant L-FABP, and rabbit antirat liver SCP-2 antibodies were prepared using specific pathogen-free New Zealand White rabbits as described in Harlow and Lane (24). Antibodies directed against one protein were not crossreactive with the other proteins (25).

Cell culture. The fetal rat colon cell line immortalized with the SV40 large T antigen (FRC/TEX/CL4D), and the *ras* and *src* transformants of CL4D (FRC/TEX/*RAS* and FRC/TEX/*SRC*, respectively) were kindly provided by Dr. Ian Summerhayes (4). Stock cultures were grown in a humidified atmosphere of 5% CO_2 at 37°C in William's Medium E supplemented with 9% fetal bovine serum, 10^{-8} M insulin, 20 µg/L hydrocortisone, 120 µg/L transferrin, penicillin G 100 units/mL, and streptomycin 100 µg/mL.

Measurement of protein turnover. Rates of protein synthesis and degradation were measured as previously described (26). Briefly, the cells were passaged into 24-well multiwell plates and incubated in DMEM as described below. At 18 h after addition of 3 mM butyrate, the medium was changed to one of exactly the same formulation, but also containing 10 µCi/mL and 2 mM ^{3}H-leucine, and the cells were pulse-labeled for 3 h. Then the monolayer was rinsed three times with Earle's salts containing 2 mM leucine before the cells were incubated in DMEM containing 2 mM leucine for the 3-h protein breakdown measurement period. Rates of protein degradation (percentage in 3 h) are reported as the percentage of total radioactivity soluble in 10% trichloroacetic acid. Rates of protein synthesis were measured using exactly the same protocol except that ^{3}H-leucine (10 µCi/mL and 2 mM) was added during the 3-h measurement period. Rates of protein synthesis are reported as nmol leucine incorporated per 3 h per mg protein.

Preparation of cell samples for protein determination, gel electrophoresis, and immunoblotting. Cells were plated at 3×10^5 cells/100 mm Petri dish in DMEM containing 9% fetal bovine serum, 10^{-8} M insulin, 20 µg/L hydrocortisone, 120 µg/L transferrin, penicillin G 100 units/mL, and streptomycin 100 µg/mL. After 24 h, the medium was changed to one containing 1% serum, and 24 h later to one containing 9% serum with and without 3 mM sodium butyrate. After a further 24 h, when the cells were in logarithmic growth, the medium was

aspirated, the monolayer was rinsed twice with cold phosphate-buffered saline (PBS), the cells were scraped into 10 mL cold PBS and centrifuged at 100 × *g* for 3 min, and the cell pellet stored at −75°. Subsequently the pellet was thawed and 0.1 to 0.5 mL of a buffer containing 500 µM PMSF, 1 mM EDTA, 5 µg/mL leupeptin, and 15 µg/mL pepstatin-A was added. The pellet was then vortexed well followed by sonication for a total of 30 s at 35% maximum speed with a Sonic Dismembrator Model 300 (Fisher, Pittsburgh, PA) to lyse the cells.

Protein determination. Total protein was determined by the method of Bradford (27). Three aliquots of each cell lysate were analyzed and the mean total protein content determined using bovine serum albumin as the standard.

Tricine gel electrophoresis and immunoblotting. An aliquot of each sample was prepared for electrophoresis by the addition of an equal volume of buffer containing glycerol, 2-mercaptoethanol, 100 mM tris-HCl, SDS, and Coomassie G250. Samples (7.5–150 µg protein/lane) were electrophoresed in 16.5% tricine gels as described by Schagger and von Jagow (28). Proteins were transferred to PVDF membranes, the membranes allowed to air-dry 2 h at room temperature, and immunoblotting was performed. Briefly, the blots were blocked with 5% nonfat dry milk in tris-buffered saline + 0.05% Tween 20 (TBST), incubated with a 1:1000 dilution of the appropriate primary antibody (rabbit anti-ACBP, rabbit anti-I-FABP, rabbit anti-L-FABP, rabbit anti-SCP-2, or mouse anti-actin) in TBST for 12–14 h. After washing with TBST three times, one of two techniques was utilized, depending upon the sensitivity desired. In general, blots were incubated with horseradish peroxidase conjugated goat antirabbit IgG, washed with tris-buffered saline (TBS) three times and 4-chloro-1-naphthol used as the substrate. For greater sensitivity, blots were incubated with 1.5 µg/mL biotinylated goat antirabbit IgG or biotinylated goat antimouse IgG for 2–3 h, washed with TBST three times, incubated with avidin-biotin complex-alkaline phosphatase 1–3 h, washed three times with TBS, and developed with Vector black substrate kit.

Quantitation of proteins on immunoblots. Photographs of immunoblots were obtained using an IS-500 computerized gel documentation system (Alpha-Innotech, San Leandro, CA). From each image, protein bands from the colon cell preparations in each lane were quantitated by comparing to known amounts of protein standards loaded on the same blot by using an NIH Image 1.60 program on a Power MacIntosh 8100/80AV computer (written by Wayne Rasband at the U.S. National Institute of Health and available from the Internet by anonymous FTP from zippy.nimh.gov or on a floppy disk from NYIS, 5285 Port Royal Rd., Springfield, VA). Protein was expressed as ng/µg sample.

Statistical analysis. Three cell lines in the absence or presence of butyrate were analyzed for each of the following categories: control (immortalized colon cells), *ras*-transformed colon cells, and *src*-transformed colon cells. ACBP, I-FABP, L-FABP, SCP-2, and actin protein content were determined in each cell line (see above), and the mean ± SE determined for each category (n = 4–7 experiments for ACBP; n = 2–3 experiments with I-FABP, L-FABP, SCP-2, and actin). An unpaired Student's t-test was performed for comparison of control immortalized colon cells vs. *ras*- and *src*-transformed colon cells and for comparison of the effects of butyrate treatment on each cell line.

RESULTS

The immortalized control cell line, CL4D, grew with a doubling time of approximately 1 d to a density of 1×10^5 cells per cm^2 (approximately 50 µg total cell protein/cm^2). The *ras* and *src* transformed cell lines grew more rapidly (doubling times of 0.5–0.7 d) to cell densities 2–3 times those of the control cell line. In keeping with their higher rates of proliferation, the two transformed cell lines also had higher rates of protein synthesis and lower rates of protein breakdown (see below) as has been found previously for other comparisons of normal and transformed cells (26).

The expression of ACBP in control, *ras*-transformed, and *src*-transformed colon cell lines was determined as described in the Materials and Methods section. Protein content was determined for each band at 10 kDa in each lane by comparison to a panel of standards containing a known amount of ACBP, applied on the same immunoblot. ACBP was expressed in relatively high amounts in the control cells, 0.75 ± 0.13 ng/µg cell protein, similar to the level expressed in colon and intestinal mucosa (reviewed 8). Transformation resulted in significantly higher expression of ACBP (1.30 ± 0.11 and 1.11 ± 0.10 ng/µg cell protein for *ras*- and *src*-transformed cell lines, respectively; n = 7). *Ras* transformation resulted in a 1.8-fold increase ($P < 0.01$) in ACBP protein levels as compared to controls while *src* transformation resulted in a 1.5 fold increase ($P < 0.05$). A representative immunoblot is shown in Figure 1A.

Both L-FABP and I-FABP were poorly expressed in immortalized rat colon cells *in vitro* where actual protein levels were measured by immunoblotting (Fig. 1B,C), similar to that obtained for colonic mucosal epithelial cells (13). Since *ras* and *src* transformation increased the level of ACBP expression in our study, it was important to determine the effect of transformation on I-FABP and L-FABP expression. Neither *ras* nor *src* transformation increased the expression of L-FABP or I-FABP. For all cell lines, up to 150 µg of cell homogenate was loaded per lane, and 0.05 ng purified protein standard was the lowest detectable level on each blot (Fig. 1B,C). Therefore, both control and transformed cell lines contained less than 0.6 picograms of L-FABP and I-FABP per microgram of homogenate protein.

The expression of SCP-2 in control, *ras*-transformed, and *src*-transformed cell lines was examined. Each immunoblot was analyzed as described for ACBP. SCP-2 expression in control immortalized colon cells was relatively high, 0.42 ± 0.02 ng/µg cell protein, and similar to that of intestine (17). The level of SCP-2 expression was significantly decreased by *ras* and *src* transformation compared to control cell lines. *Ras*

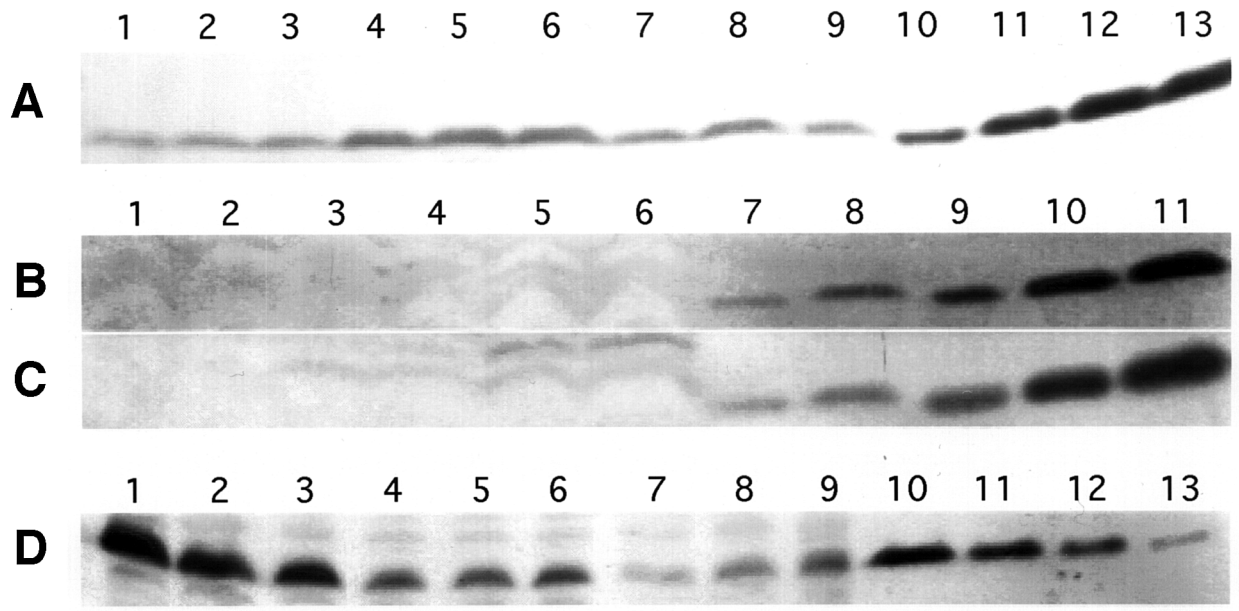

FIG. 1. Representative immunoblots of ACBP, I-FABP, L-FABP, and SCP-2 protein expression in rat colon epithelial cell lines. The cells were grown, protein was extracted, and immunoblotting performed as described in the Materials and Methods section. A: Lanes 1–3 were loaded with 25 µg of protein from control cell lines, 4–6 from *ras*-transformed cell lines, and 7–9 from *src*-transformed cell lines. Lanes 10–13 contain 5, 10, 15, and 20 ng, respectively, of purified rat liver ACBP. B: I-FABP protein expression in rat colon epithelial cell lines. Lanes 1 and 2 were loaded with 75 µg of protein from control cell lines, 3 and 4 from *ras*-transformed cell lines, and 5 and 6 from *src*-transformed cell lines. Lanes 8–12 contain 0.05, 0.1, 0.2, 0.5, and 1 ng, respectively, of purified I-FABP. C: L-FABP protein expression in rat colon epithelial cell lines. Lanes 1–6 were loaded the same as in B above. Lanes 8–12 contain 0.05, 0.1, 0.2, 0.5, and 1 ng, respectively, of purified L-FABP. D: SCP-2 protein expression in rat colon epithelial cell lines. Lanes 1–3 were loaded with 35 µg of protein from control cell lines, 4–6 from *ras*-transformed cell lines, and 7–9 from *src*-transformed cell lines. Lanes 10–13 contain 12, 9, 6, and 3 ng, respectively, of purified recombinant SCP-2.

transformation resulted in a 55% decrease ($P < 0.005$) in SCP-2 protein levels while *src* transformation resulted in a 67% decrease ($P < 0.005$) in SCP-2 expression, from 0.42 ± 0.02 to 0.19 ± 0.02 and 0.14 ± 0.03 ng/µg cell protein, respectively ($n = 2$). A representative immunoblot is shown in Figure 1D.

Butyrate treatment (3 mM) decreased cell proliferation much more in transformed cell lines than control (Fig. 2). In the control cell lines the decrease was modest with a 1.1-fold increase in the doubling time, whereas in the transformed cell lines doubling times increased 1.7-fold and 2.5-fold in the *ras*- and *src*-transformed cell lines, respectively. These changes in proliferation were evident at 24 h. In keeping with the changes in proliferation, butyrate also significantly decreased rates of protein synthesis ($P < 0.05$) and significantly increased rates of protein breakdown ($P < 0.05$) in all cell lines when compared to the respective untreated cell lines (Table 1). The effect of butyrate was particularly marked in the *src*-transformed cell lines.

To further assess the effects of butyrate upon general protein synthesis and to determine if decreased protein synthesis might account for changes in ACBP, L-FABP, I-FABP, or SCP-2 expression, the expression of actin was assessed. Actin is a component of the cytoskeleton of cells and considered to be a housekeeping protein normally expressed in constant levels within the cell. The levels of actin were: 4.2 ± 0.4, 2.1 ± 0.2, and 1.3 ± 0.1 ng/µg cell protein for immortalized, *ras*-transformed, and *src*-transformed cell lines, respectively. The levels of actin were higher in control cells and decreased by more than 50% in the transformed cell lines (Fig. 3). These data are consistent with previous reports in which actin expression and cytoskeletal organization were decreased in different types of transformed and neoplastic cells as compared to normal cells (29,30). Treatment with butyrate resulted in a mild decrease in actin expression, but this change was not significant in cell lines treated with butyrate compared to untreated cell lines (Fig. 3). The levels of actin were 3.2 ± 0.45,

TABLE 1
3-Hour Pulse-Chase After Treatment with Butyrate (for 24 h)[a]

Cell line	Protein synthesis	Protein degradation
CL4D	30.0 ± 1.1	12.12 ± 0.15
CL4D + butryate	24.7 ± 1.0*	14.09 ± 0.36*
ras	31.6 ± 1.0	11.66 ± 0.22
ras + butyrate	27.9 ± 1.1*	13.13 ± 0.13*
src	31.2 ± 0.9	11.62 ± 0.17
src + butyrate	20.4 ± 2.0*	13.45 ± 0.15*

[a]Protein synthesis is nmol leucine incorporated/mg protein in 3 h. Protein degradation is the percentage of total protein degraded in 3 h (after a 3-h pulse-chase); * = significant difference from the respective cell line without butyrate ($P < 0.05$).

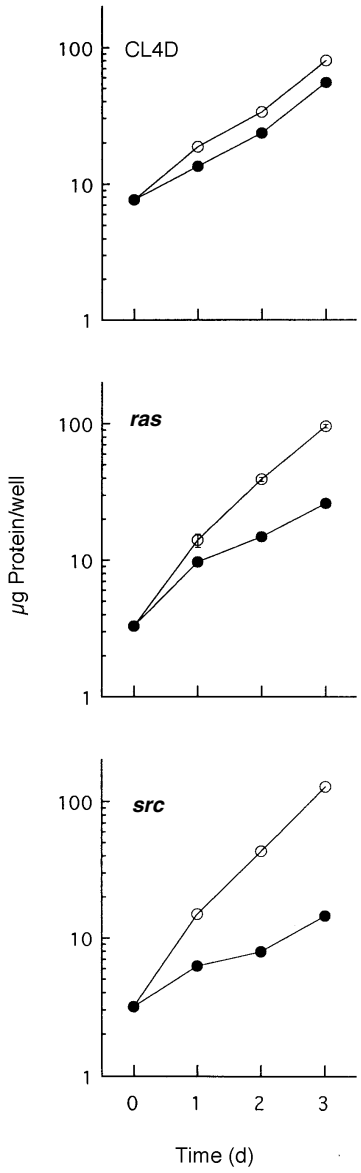

FIG. 2. Effect of butyrate on growth of rat colon epithelial cell lines. All cell lines were seeded on day −1, harvested at 24-h intervals, and protein was extracted and quantitated as described in the Materials and Methods section. The growth of untreated cell lines, ○; butyrate-treated cell lines, ●.

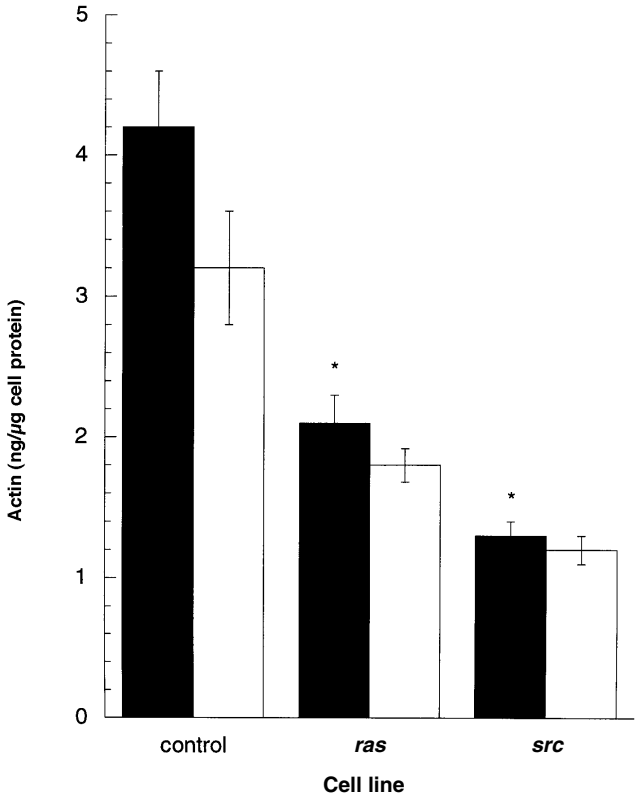

FIG. 3. Comparison of actin expression in control, *ras*-transformed, and *src*-transformed cell lines with and without butyrate treatment. The cells were grown, protein was extracted, and immunoblotting performed as described in the Materials and Methods section. Actin is expressed as ng/μg cell protein, and each bar represents the average ± SEM of three cell lines for $n = 3$ experiments. Open bars represent cell lines treated with butyrate. Solid bars represent cell lines without butyrate treatment. * = significant difference from the control cell line without butyrate treatment ($P < 0.05$). Statistical analysis was performed using an unpaired Student's *t*-test.

1.8 ± 0.12, and 1.2 ± 0.1 ng/μg cell protein in the butyrate-treated immortalized, *ras*-transformed, and *src*-transformed cell lines, respectively.

ACBP expression was examined in cell lines with and without butyrate treatment. Cells were harvested 24 h after beginning butyrate treatment and protein was determined as described in the Materials and Methods section; each immunoblot was analyzed as described in this section. Butyrate treatment resulted in decreased expression of ACBP in all cell lines. Figure 4 shows that ACBP was decreased by 40, 36, and 24% in control, *ras*-, and *src*-transformed cells treated with butyrate when compared to the respective untreated cells. Although butyrate decreased ACBP expression in all three cell lines, a statistically significant difference in ACBP expression was observed only in butyrate-treated *src*-transformed cells vs. butyrate-treated control cells. Thus, the reduction in cell proliferation and general protein synthesis observed in response to butyrate in the three cell lines was reflected in the decreased level of ACBP in response to butyrate treatment.

To determine if the effect of butyrate on inhibition of protein synthesis as exemplified by decreased expression of ACBP was a specific effect for this acyl-CoA binding protein, the level of expression of SCP-2 was examined. In contrast to ACBP, SCP-2 expression did not change in response to butyrate treatment (Fig. 5). While SCP-2 levels were decreased in *ras*- and *src*-transformed cell lines as before, butyrate treatment did not significantly alter expression of SCP-2 in either the control or transformed cells (Fig. 5). Thus, butyrate differentially modulated the expression of ACBP and SCP-2 in control and transformed colon cells. Moreover, these effects were distinct from the general inhibition of cell proliferation

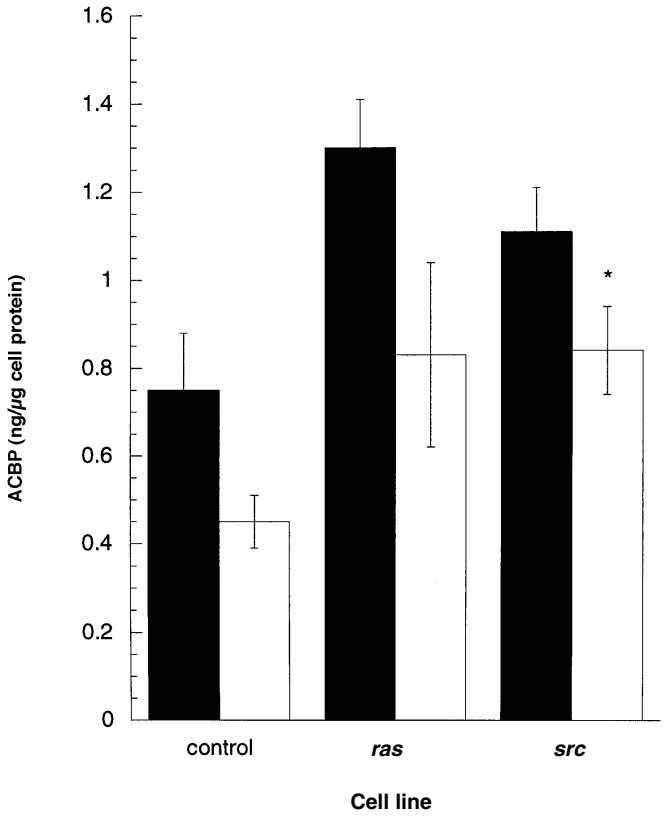

FIG. 4. Comparison of ACBP expression in control, *ras*-transformed, and *src*-transformed cell lines with and without butyrate treatment. The cells were grown, protein was extracted, and immunoblotting performed as described in the Materials and Methods section. ACBP is expressed as ng/μg cell protein, and each bar represents the average ± SEM of three cell lines; $n = 4$ experiments. Open bars represent cell lines treated with butyrate. Solid bars represent cell lines without butyrate treatment. The * indicates significant difference from control cell lines treated with butyrate ($P < 0.05$). Statistical analysis was performed using an unpaired Student's *t*-test.

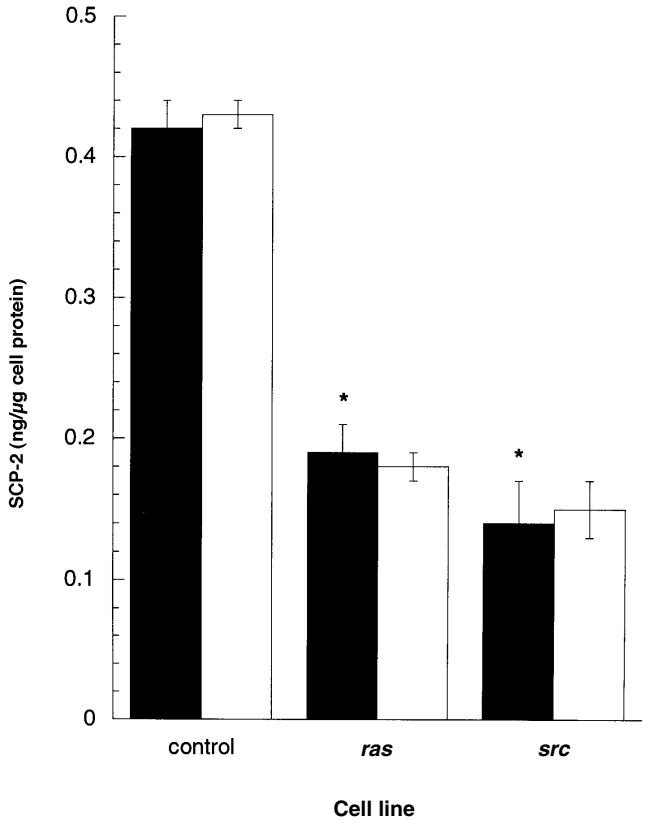

FIG. 5. Comparison of SCP-2 expression in control, *ras*-transformed, and *src*-transformed cell lines with and without butyrate treatment. The cells were grown, protein was extracted, and immunoblotting performed as described in the Materials and Methods section. SCP-2 is expressed as ng/μg cell protein, and each bar represents the average ± SEM of three cell lines, $n = 3$ experiments. Open bars represent cell lines treated with butyrate. Solid bars represent cell lines without butyrate treatment. * = significant difference from the control cell line without butyrate treatment ($P < 0.05$). Statistical analysis was performed using an unpaired Student's *t*-test.

and protein synthesis by butyrate in both control and transformed cells.

DISCUSSION

Of the four fatty acyl-CoA binding proteins examined, transformation differentially altered the expression of ACBP and SCP-2. ACBP was the only protein in which expression increased as a result of transformation with *ras* and *src* oncogenes. Increased ACBP expression has been demonstrated in a number of different human brain tumors *in vivo* such as astrocytomas, glioblastomas and medulloblastomas, and the highest expression occurred in the most anaplastic of these tumors. In contrast, ACBP levels were low to undetectable in normal brain and benign brain neoplasms such as meningiomas and pituitary adenomas (31). Other tumor cells in which ACBP has been identified include human prostatic adenocarcinoma cell lines, mouse adrenal and Leydig cell-derived tumor cell lines, rat insulinoma cell line, dimethylbenz(α)anthracene-induced mammary tumors in the rat, osteosarcoma cell line, neuroblastoma cell line, and the supernatant from a metastatic human malignant melanoma cell line (reviewed 8). However, quantitative differences in the level of ACBP expression in these tumor cells compared to control (untransformed) cells have not been determined.

The function of ACBP *in vivo* has not been fully characterized, but it may be involved in the regulation of the intracellular acyl-CoA pool and lipid metabolism, modulation of benzodiazepine receptors, regulation of glucose-induced insulin secretion, and regulation of cell growth (reviewed 8). The potential role of ACBP in cell growth of transformed colon cells *in vitro* could be explained by several possible mechanisms. First, transformed and neoplastic cells grow faster and have a higher metabolic activity than normal cells. Therefore, ACBP levels may be increased secondary to increased acyl-CoA levels and turnover within transformed cells. Second, ACBP may be involved directly in the regulation of cell growth *via* interaction with peripheral benzodiazepine receptors (PBR). PBR have been postulated to be involved in mitochondrial and cell proliferation (32). In studies

with a human Leydig-derived cell line and mouse 3T3 swiss fibroblasts, nanomolar concentrations of ACBP exerted a mitogenic effect possibly *via* interaction with PBR (33). In addition, increases in PBR were detected by immunohistochemical analysis in human brain neoplasms with increased ACBP, and increased PBR have been detected in human colon carcinomas and other tumor types (34,35).

Third, by regulating acyl-CoA transport and acyl-CoA levels within the cell, ACBP may alter cell signaling pathways through protein myristoylation and protein phosphorylation. Increased *N*-myristoyl-transferase activity has been observed in rat and human colon tumors when compared to normal colon tissue (36). Myristoylation is the covalent modification of proteins in which myristate or palmitate is covalently attached to the N terminus of cellular proteins. Many of these proteins are involved in signal transduction, and of particular interest in colon neoplasia are tyrosine kinases such as $pp60^{c\text{-}src}$ and the alpha subunit of several guanine nucleotide binding protein receptors (*ras* encodes p21, a membrane-bound G-protein) (37). Further studies will be necessary to determine how ACBP expression is involved in neoplastic transformation and its role in the regulation of cell proliferation.

Butyrate affected both cell proliferation and ACBP expression in the transformed rat colon epithelial cells. The effects of butyrate on cell proliferation in these cell lines are consistent with previous reports of its effects on other types of cells *in vitro*. However, the decreased ACBP expression upon butyrate treatment was not due to a general decrease in protein synthesis attributed to cell death, since the levels of actin and SCP-2 stayed the same in untreated and treated cells. Therefore, ACBP expression appears to be higher in the transformed, undifferentiated state in which cells are rapidly proliferating. Butyrate treatment apparently resulted in induction of a more differentiated state with decreases in DNA synthesis and cell proliferation and a simultaneous decrease in ACBP expression.

Although both L-FABP and I-FABP are expressed in intestinal mucosal epithelial cells, the level of expression is highest in the duodenum and jejunum, but decreases markedly in the colon (13). L-FABP and I-FABP were undetectable in the cell lines examined. Both L-FABP and I-FABP expression are markedly reduced in several liver and intestinal tumors, including human and rat colon carcinomas, as well as other types of transformed cell lines in culture (12, 19,20,38–40). Caco-2 and HT-29 cell lines (derived from two different human colon carcinomas), and several mouse- and rat-derived colon carcinoma cell lines did not express detectable levels of I-FABP mRNA (39,40) while only the Caco-2 cell line expressed detectable levels of L-FABP mRNA (40).

In contrast to ACBP expression, SCP-2 expression decreased in *ras*- and *src*-transformed cell lines. This is the first report of changes in SCP-2 expression in colon epithelial cells, but the findings are consistent with other studies in different systems. Rat hepatoma cells express lower levels of SCP-2 than normal rat hepatocytes, and transformed cells also showed decreased expression of SCP-2 (17,41). In addition, changes in the expression of SCP-2 have been reported in human adrenocortical tissue. A significant decrease in SCP-2 expression occurred in adrenocortical carcinomas when compared to normal tissue and benign neoplasms (42). Butyrate treatment did not affect SCP-2 expression in control or transformed cells. Therefore, SCP-2 does not appear to be associated with cell proliferation in this cell type and was not affected by changes in differentiation induced by this agent.

Actin expression decreased 50% or more in the transformed rat colon epithelial cell lines which is consistent with other reports in different types of cells. Actin has been shown to be a sensitive marker of transformation in NIH 3T3 fibroblasts (29). Transformation results in reduced synthesis of smooth muscle α-actin and various actin-binding proteins which results in changes in cytoskeletal organization and the transformed phenotype of these cells (29). Several other cell lines such as human fibrosarcoma, osteosarcoma, and MOLT-4 cells derived from a T-cell leukemia exhibit decreases in the level of β-actin (30). SCP-2 expression in the rat colon epithelial cell lines was approximately 10-fold lower than actin and transformation resulted in a decrease in SCP-2 similar to that of actin. Therefore, comparison of the ratio of actin to SCP-2 in control vs. transformed cell lines resulted in a similar ratio of 10, 11, and 9.3 in control, *ras*-transformed, and *src*-transformed cell lines, respectively. However, since ACBP expression increased in the transformed rat colon epithelial cells, the ratio of ACBP to actin increased significantly as a result of transformation (from 0.18 in control cell lines to 0.62 and 0.85 in the *ras*- and *src*-transformed cell lines, respectively) (Table 2). Likewise, comparison of the ratio of ACBP to SCP-2 expression in control vs. transformed cell lines resulted in a ratio of 1.8, 6.8, and 7.9 in control, *ras*-transformed, and *src*-transformed cell lines, respectively. Butyrate treatment decreased ACBP expression but did not affect actin or SCP-2 levels significantly. This resulted in ratios of ACBP to these proteins that were lower than those of the untreated cell lines. However, *ras* transformation with and without butyrate treatment resulted in an approximately 3.8–4.2-fold increase in the ratio of ACBP to SCP-2 while *src* transformation resulted in a 4.4–5.1-fold increase (Table 2). Therefore, changes in the ratio of SCP-2 to ACBP expression appear to be a sensitive marker of tranformation in the rat model of immortalized colon epithelial cells.

TABLE 2
Comparison of the Ratio of Expression of Actin, SCP-2, and ACBP With and Without Butyrate Treatment

	Without butyrate		With butyrate	
Cell line	ACBP/actin	ACBP/SCP-2	ACBP/actin	ACBP/SCP-2
Control	0.18	1.8	0.14	1.1
ras-transformed	0.62[a]	6.8[a]	0.46[a]	4.6[a]
src-transformed	0.85[a]	7.9[a]	0.70[a]	5.6[a]

[a]Significant difference from the respective control.

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Fatty Acid Composition of Lymphocytes and Macrophages from Rats Fed Fiber-Rich Diets: A Comparison Between Oat Bran- and Wheat Bran-Enriched Diets

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ABSTRACT: The effect of oat bran- (OBD) and wheat bran-enriched diets (WBD) on fatty acid composition of neutral lipids and phospholipids of rat lymphocytes and macrophages was investigated. In neutral lipids of lymphocytes, OBD reduced the proportion of palmitoleic acid (48%), whereas WBD reduced by 43% palmitoleic acid and raised oleic (18%), linoleic (52%), and arachidonic (2.5-fold) acids. In neutral lipids of macrophages, OBD increased palmitic (16%) and linoleic (29%) acids and slightly decreased oleic acid (15%). The effect of WBD, however, was more pronounced: It reduced myristic (60%), stearic (24%) and arachidonic (63%) acids, and it raised palmitic (30%) and linoleic (2.3-fold) acids. Neither OBD nor WBD modified the composition of fatty acids in phospholipids of lymphocytes. In contrast, both diets had a marked effect on composition of fatty acids in macrophage phospholipids. OBD raised the proportion of myristic (42%) and linoleic (2.4-fold) acids and decreased that of lauric (31%), palmitoleic (43%), and arachidonic (29%) acids. WBD increased palmitic (18%) and stearic (23%) acids and lowered palmitoleic (35%) and arachidonic (78%) acids. Of both cells, macrophages were more responsive to the effect of the fiber-rich diets on fatty acid composition of phospholipids. The high turnover of fatty acids in macrophage membranes may explain the differences between both cells. The modifications observed due to the effects of both diets were similar in few cases: an increase in palmitic and linoleic acids of total neutral lipids occurred and a decrease in palmitoleic and arachidonic acids of phospholipid. Therefore, the mechanism involved in the effect of both diets might be different.

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Volatile short-chain fatty acids (VSCFA) comprise the major solute fraction of fecal water, being produced by fermentation of water-soluble fiber by anaerobic bacteria normally resident in the large bowel (1). Acetic, propionic, and butyric acids are the predominant forms of the VSCFA in the gastrointestinal tract (2). Since dietary fiber reduces the incidence of colon cancer in humans (3), the possible role of these fatty acids in cell growth and differentiation has been investigated. It is widely accepted that butyrate causes a marked inhibition of the growth of several tumor cell lines in culture (4–6). A recent study from our laboratory has shown that propionate, at concentrations varying from 1 to 5 mmol/L, also inhibits the proliferation of tumor (HeLa-155 and KB-372) and normal (MA-104) cell lines *in vitro* (7). In addition, it has been found that propionate can influence the proliferation of lymphocytes: It stimulates proliferation at low concentrations (0.04 and 0.1 mmol/L) but inhibits proliferation at concentrations above 1 mmol/L (8).

Lymphocytes and macrophages utilize glucose and glutamine at high rates, but these substrates are only partially oxidized: Glucose is converted to lactate and glutamine to glutamate, aspartate, and lactate (9). Pyruvate generated by either substrate is not completely oxidized, but some of it may be converted to acetyl-CoA for lipid synthesis in these cells (10,11). Lipogenesis has been recognized to be important for the process of cell proliferation to take place. However, there is evidence that either lymphocytes (rapidly dividing cells) and macrophages (nondividing cells) export lipids produced from pyruvate (12). Propionate does not affect the rates of glycolysis or glutaminolysis, but it decreases the rate of lipogenesis in incubated lymphocytes (12), which may explain the inhibitory effect of the VSCFA on lymphocyte proliferation. Propionate at a concentration of 40 mmol/L decreases the incorporation of [1-^{14}C]-palmitate into phospholipids (86%), triacylglycerol (87%), and cholesterol ester (90%) in 24-h cultured lymphocytes (13). A similar effect of propionate in macrophages has been reported (14); this VSCFA inhibits the incorporation of [1-^{14}C]-palmitate into phospholipids, cholesterol, cholesterol ester and triacylglycerol, and the incorporation of [3-^{14}C]-pyruvate into phospholipids. The rate of phospholipid turnover and fatty acid incorporation in macrophage membranes is very high, probably owing to their

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Abbreviations: MTBE, methyltertiarylbutylether; OBD, oat bran-enriched diet; VSCFA, volatile short-chain fatty acids; WBD, wheat bran-enriched diet.

various endocytic and exocytic activities (15). It has been shown that a disproportional incorporation of saturated and polyunsaturated fatty acids into macrophages is able to regulate their ability to adhere to surfaces and to perform phagocytosis (16,17). Therefore, cellular deacylation processes, which remove the CoA moieties from the fatty acyl-CoA and so their ability to be incorporated into phospholipids, might play an important role for the normal function of macrophages. Propionate raises the activities of palmitoyl-CoA and oleoyl-CoA deacylases in macrophages (14) and lymphocytes (13). These findings support the proposition that this VSCFA might cause important changes in fatty acid composition of these cells by inhibiting lipogenesis and altering the deacylation process.

The information above and the current trend to encourage consumption of high-fiber diets led us to investigate the effect of fiber-rich diets on the fatty acid composition of total neutral lipids and phospholipids of lymphocytes and macrophages. These cells present high turnover of fatty acids, which might ensure the high proliferative capacity of lymphocytes and the endocytic and secretory activities of macrophages. Water-soluble fibers are the main source of VSCFA (18,19), therefore, fiber-rich diets presenting different soluble/insoluble fiber ratios were used: oat bran (0.3) and wheat bran (0.14).

MATERIALS AND METHODS

Animals and diet preparation. Just-weaned male Wistar rats weighing 50–60 g were obtained from the Animal House of Instituto de Ciências Biomédicas, USP, (São Paulo, Brazil). During the experiment, the rats were kept in groups of 5 at 23°C with a 12-h light/12-h dark cycle; lights on at 7:00. The rats were divided into three groups: (i) control—fed a regular diet (composition shown in Table 1 and similar to that reported by Anderson (20), (ii) oat bran-enriched diet (OBD)—prepared by addition of 300 g of oat bran in 1000 g of regular diet, and (iii) wheat bran-enriched diet (WBD)—prepared by addition of 300 g of wheat bran in 1000 g of regular diet. The soluble/insoluble fiber ratio was 0.22, 0.30, and 0.14 for control, OBD, and WBD, respectively. The fatty acid compositon of the three diets was similar to that shown in Table 1. The rats were fed the respective diet for 8 wk and, during this time, body weight, food intake, and fecal output were evaluated weekly. After this period, the rats were killed by decapitation and total blood, lymph nodes lymphocytes, and resident peritoneal macrophages were collected. Animals were always killed between 8:00 and 11:00 h.

Chemicals. Solvents were obtained from Fisons Scientific Apparatus (Loughborough, Leics., United Kingdom) and were redistilled before use. An ethereal solution of diazomethane was prepared from Diazald (Aldrich Chemical Co., Gillingham, Dorset, United Kingdom).

Preparation of lymphocytes and macrophages. Mesenteric lymph nodes were dissected from rats and lymphocytes were prepared as previously described (21).

For preparation of resident macrophages, the rats were intraperitoneally injected with 6 mL of cold saline 0.9% and the cells obtained as previously described (22).

TABLE 1
Basic Composition of the Regular Diet[a]

Component (g/kg of diet)	
Protein (casein)	180
Fat	40
Carbohydrates	455
Minerals and vitamins	80
Calcium	18
Phosphorus	8
Insoluble fiber	180
Soluble fiber	39
Fatty acid composition (g/100 g)	
Lauric acid	n.d.
Myristic acid	0.63
Palmitic acid	17.03
Palmitoleic acid	0.50
Stearic acid	2.62
Oleic acid	26.60
Linoleic acid	49.39
Linolenic acid	3.23
Arachidonic acid	n.d.
Vitamin mixture (units/100 g)	
Vitamin A	20,000 UI
Vitamin D_3	6,600 UI
Vitamin E	30.0 mg
Vitamin K	6.0 mg
Vitamin B_{12}	10.0 mcg
Vitamin B_2	8.0 mg
Niacin	95.0 mg
Pantothenic acid	24.0 mg
Thiamine	4.0 mg
Choline	2,000 mg
Piridoxin	6.0 mg
Biotin	0.1 mg
Folic acid	
Trace metals (units/100 g)	0.5 mg
Manganese	50.0 mg
Iodine	2.0 mg
Iron	65.0 mg
Zinc	35.0 mg
Copper	26.0 mg
Antioxidant (butylated hydroxytoluene–BHT)	100.0 mg

[a]Vitamin K was purchased from Roche Laboratories. BHT was obtained from Shell Chemical (Houston, TX).
n.d. = Not detected; UI, unsaturation index.

Lipid extraction and analysis. Macrophages and lymphocytes were resuspended in a small volume of phosphate buffered-saline and sonicated (three times of 15 s each at an amplitude of 12 μm) in an MSE sonicator (LAB-LINE Instruments Inc., Melrose Park, IL). Lipid was extracted with chloroform/methanol solution (2:1, vol/vol). Neutral lipids and phospholipids were separated using Sep-Pak columns (Waters Corporation, Milford, MA), as described by Hamilton and Comai (23). Columns were flushed with 12 mL hexane and the sample, dissolved in 1 mL methyl tertiarybutylether (MTBE), was applied. Neutral lipids were eluted using 10 mL MTBE/methanol/0.001 M ammonium acetate (100:0.2, vol/vol), and phospholipids were eluted using 10 mL

MTBE/methanol/0.001 M ammonium acetate (5:8:2, by vol). Fatty acids were prepared by overnight saponification at 70°C in methanolic 0.5 M KOH. Samples were neutralized using concentrated sulfuric acid, and fatty acids were extracted in ethyl acetate and methylated by reaction with diazomethane (24). A similar procedure has been employed in a previous study (17). Fatty acid methyl esters (dissolved in methyl acetate) were separated by gas chromatography in a Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA) fitted with a 50 m × 0.3 mm bonded phase OV-1 fused-silica capillary column, film thickness 0.52 μm. Helium at 2.0 mL/min was used as the carrier gas, and the split/splitless injector was used in the split mode, with a split ratio of 15:1. Injector and detector temperatures were both 300°C, and the column oven temperature was programmed from 130 to 380°C at 2°C/min. The separation was recorded with a Servoscribe chart recorder (Hewlett-Packard), and quantitative data were recorded with a Hewlet-Packard 3390A recording integrator. Fatty acids were identified by comparison with standards run previously.

Data presentation and statistics. All data are presented as mean ± SD for the indicated number of samples. Statistical significance was determined using the two-tailed unpaired Student's test; a value for P of less than 0.05 was considered to indicate a statistically significant difference.

RESULTS AND DISCUSSION

The body weight gain, food intake, and output of feces were not different among the groups (data not shown).

The fatty acid composition of the blood was not markedly affected by OBD or WBD (data not shown). In fact, compared to control diet, the only significant difference was a 68% increase in the proportion of arachidonic acid in the serum of rats fed OBD. This increase in the proportion of arachidonic acid may reflect a modification of its metabolism and so of the production of eicosanoids (25), which could be important for regulating cell functions (26).

In contrast to this unique change found in the fatty acid composition of the blood, the fatty acid composition of lymphocytes and, more particularly, in macrophages was substantially modified by both fiber-rich diets (Tables 2–4). The OBD reduced the proportion of palmitoleic acid (by 48%) in the total neutral lipids of lymphocytes (Table 2). In contrast, the WBD had a pronounced effect on fatty acid composition of neutral lipids of lymphocytes: this diet reduced by 43% palmitoleic acid and raised the proportions of oleic (by 18%), linoleic (by 52%), and arachidonic (2.5-fold) acids. In total neutral lipids of macrophages (Table 3), the OBD increased the proportion of palmitic (by 16%) and linoleic (by 29%) acids and diminished that of oleic acid (by 15%). The effect

TABLE 2
Fatty Acid Composition of Total Neutral Lipids of Mesenteric Lymph Node Lymphocytes

		Groups	
Fatty acids	Control	Oat bran	Wheat bran
Lauric acid	1.40 ± 0.67	1.18 ± 0.33	0.67 ± 0.33
Myristic acid	4.24 ± 1.39	3.73 ± 0.56	3.84 ± 0.85
Palmitic acid	43.53 ± 6.17	42.98 ± 6.70	39.52 ± 7.00
Palmitoleic acid	4.76 ± 0.52	2.46 ± 2.07*	2.71 ± 0.64*
Stearic acid	13.11 ± 3.74	17.04 ± 4.35	12.12 ± 2.23
Oleic acid	18.46 ± 1.40	19.94 ± 5.18	21.71 ± 0.94*
Linoleic acid	9.74 ± 2.99	8.77 ± 2.04	14.77 ± 3.38*
Linolenic acid	3.82 ± 1.35	3.04 ± 1.33	2.33 ± 0.43
Arachidonic acid	0.94 ± 0.30	0.86 ± 0.49	2.33 ± 0.43*

*($P < 0.05$) for comparison with the control group.
[a]From rats fed either soluble (oat bran) or insoluble (wheat bran) fiber-rich diets for 8 wk. The results are presented as mean ± SD of five rats.

TABLE 3
Fatty Acid Composition of Total Neutral Lipids of Intraperitoneal Macrophages[a]

		Groups	
Fatty acids	Control	Oat bran	Wheat bran
Lauric acid	n.d.	n.d.	n.d.
Myristic acid	6.70 ± 0.84	5.72 ± 0.76	2.70 ± 0.19*
Palmitic acid	34.34 ± 1.49	39.66 ± 1.73*	44.65 ± 1.19*
Palmitoleic acid	2.43 ± 0.47	2.21 ± 0.35	2.03 ± 0.29
Stearic acid	19.00 ± 1.26	19.31 ± 1.14	14.41 ± 0.96*
Oleic acid	25.85 ± 2.38	22.08 ± 0.63*	25.25 ± 0.88
Linoleic acid	3.50 ± 0.50	4.50 ± 0.58*	7.92 ± 1.02*
Linolenic acid	n.d.	n.d.	n.d.
Arachidonic acid	8.18 ± 0.96	6.86 ± 1.59	3.04 ± 0.87*

*($P < 0.05$) for comparison with the control group; n.d. = not detected.
[a]From rats fed either soluble (oat bran) and insoluble (wheat bran) fiber-rich diets for 8 wks. The results are presented as mean ± SD of five rats.

TABLE 4
Fatty Acid Composition of Total Phospholipids of Peritoneal Macrophages[a]

Fatty acids	Control	Groups	
		Oat bran	Wheat bran
Lauric acid	3.58 ± 0.72	2.46 ± 0.67*	3.86 ± 0.51
Myristic acid	7.04 ± 0.33	10.00 ± 0.99*	7.99 ± 1.22
Palmitic acid	35.21 ± 0.41	36.86 ± 1.09	41.43 ± 2.89*
Palmitoleic acid	1.90 ± 0.36	1.09 ± 0.06*	1.23 ± 0.05*
Stearic acid	18.60 ± 1.48	18.85 ± 1.63	22.94 ± 1.65*
Oleic acid	14.84 ± 0.55	13.51 ± 3.43	14.42 ± 2.14
Linoleic acid	2.34 ± 0.52	5.58 ± 1.94*	4.49 ± 1.94
Linolenic acid	n.d.	n.d.	n.d.
Arachidonic acid	16.33 ± 0.83	11.65 ± 2.79*	3.64 ± 0.83*

*($P < 0.05$) for comparison with the control group; n.d. = not detected.
[a]From rats fed either soluble (oat bran) or insoluble (wheat bran) fiber-rich diets for 8 wk. The results are presented as mean ± SD of five rats.

of the WBD was more pronounced. This diet reduced the proportions of myristic (by 60%), stearic (by 24%), and arachidonic (by 63%) acids, and it raised that of palmitic (by 30%) and linoleic (2.3-fold) acids. Both fiber-rich diets modified the fatty acid composition of the total neutral lipids of macrophages, whereas for lymphocytes a marked effect was observed after feeding the WBD only. The effect of WBD on macrophages was more pronounced than that of OBD.

OBD and WBD did not modify the fatty acid composition of phospholipids of lymphocytes (data not shown). In contrast, both diets had a marked effect on fatty acid composition of macrophage phospholipids (Table 4). The OBD increased the proportions of myristic (by 42%) and linoleic (2.4-fold) acids and decreased that of lauric (by 31%), palmitoleic (by 43%), and arachidonic (by 29%) acids. The WBD increased the proportion of palmitic (by 18%) and stearic (by 23%) acids and decreased the proportions of palmitoleic (by 35%) and arachidonic (by 78%) acids.

The fatty acid composition of macrophage phospholipids was more sensitive to the effect of fiber-rich diets than that of lymphocytes. The OBD did not cause any significant effect on the fatty acid composition of the neutral lipids or phospholipids of lymphocytes (data not shown). The high turnover of fatty acids in macrophage membranes may explain the differences between cell types. The rate of phospholipid turnover in macrophages is very high, probably owing to their various endocytic and exocytic activities (15) so that the rate of incorporation of fatty acids into macrophage membranes might be more readily achieved than incorporation into lymphocyte membranes (27). The changes in the fatty acid composition of phospholipids of macrophages (Table 4) were different from those observed for the total neutral lipids (Table 3). These observations led us to believe that fiber-rich diets might alter the turnover of fatty acids in membrane phospholipids. Whether this effect occurred through the deacylation process remains to be investigated.

Concluding remarks. The OBD, which has a soluble/insoluble fiber ratio of 0.3 compared with 0.14 for the WBD, induced marked changes in the fatty acid composition of neutral lipids and phospholipids of macrophages. However, the WBD, although having a lower soluble/insoluble fiber ratio, also caused marked changes in the fatty acid composition of neutral lipids and phospholipids of macrophages. Both diets increased the proportion of palmitic and linoleic acids in total neutral lipids and decreased the proportion of palmitoleic and arachidonic acids in phospholipids. The mechanism involved in each case, however, might be different.

Linoleic acid is the metabolic precursor of arachidonic acid. The arachidonic acid/linoleic acid ratio of lymphocyte phospholipids was decreased by WBD: 0.3 against 0.7 for the control group. In phospholipids of macrophages, both fiber-rich diets caused a decrease of arachidonic acid/linoleic acid ratio: 7.0 for control, 2.1 for OBD, and 0.8 for WBD. These findings led us to postulate that both fiber-rich diets, particularly the one enriched with wheat bran, might affect the biosynthesis of prostaglandins and thus macrophage function. An increase in the proportion of linolenic acid, a well-known reducer of intracellular arachidonic acid levels (28), does not seem to play a role in these findings, since it was not different among the groups.

The high production of VSCFA from the fermentation of soluble fibers is not the sole mechanism by which fiber-rich diets modify the fatty acid composition of macrophages. Insoluble fibers may affect the absorption of certain fatty acids or even the formation of chylomicrons. Further studies are needed to identify both the mechanism by which diets rich in soluble and insoluble fibers alter the fatty acid composition of cells of the immune system and the functional consequences of such changes.

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Dietary Effect of a Symmetrical Triacylglycerol, 1,3-Biseicosapentaenoyl-2-γ-linolenoyl Glycerol, on Fatty Acid Composition of Guinea Pigs

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ABSTRACT: The dietary effect of 1,3-biseicosapentaenoyl-2-γ-linolenoyl glycerol (STG) on the fatty acid composition of guinea pigs was examined and compared with that of an eicosapentaenoic acid ethyl ester (EPA-E) and of a soybean oil (SBO) diet. In terms of content of plasma lipid, EPA-E had a greater hypolipidemic effect than STG. On the other hand, in terms of EPA incorporation, contents of EPA in liver lipid were almost the same in the STG and EPA-E groups. Considering that the amount of EPA administered in the EPA-E group was almost 1.5 times that of the STG group, EPA may be absorbed more effectively as the glycerol ester than as the ethyl ester in guinea pigs. In all the tissue lipids, the STG group had a higher unsaturation index (UI) than the EPA-E group even though there is a lower UI in the STG diet than the EPA-E diet. These results suggest that greater amounts of desaturase products as a whole were synthesized in the STG group than in the other two groups. The dihomo-γ-linolenic acid/arachidonic acid (DGLA/AA) ratio in plasma total lipids in the STG group was 3.5 times that of SBO group, and the DGLA/AA ratio in the EPA-E group was half that of the SBO group. In liver lipid, the ratios of DGLA/AA and EPA/AA in the STG group were 0.687 and 0.488 (phosphatidylcholine fraction) and 0.237 and 0.752 (phosphatidylethanolamine fraction), respectively. The ratio of DGLA/AA as well as the high EPA/AA ratio obtained in the present study with the STG diet may lead to physiological alterations, including enhanced synthesis of 1- and 3-series eicosanoids.

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The nutritional value of γ-linolenic acid (GLA), the product of Δ6-desaturase from linoleic acid (LA), has been suggested by the reduced Δ6-desaturase activity that occurs in many cases like diabetes (1), cardiovascular disorder (2), viral infection (3), or aging (4). Supplementation with GLA not only bypasses the enzyme reaction from LA to GLA but also has been reported to cause stimulation of Δ6-desaturase both with n-6 (5,6) and n-3 (7) essential fatty acids (EFA) (8). As well as increasing dietary GLA itself, this stimulation caused higher levels of dihomo-γ-linolenic acid (DGLA), the precursor of PGE_1, which are considered to be anti-inflammatory compared with 2-series eicosanoids derived from arachidonic acid (AA) (9). The potential usefulness of n-3 EFA has also been under study since the first epidemiological report on the lower incidence of coronary heart diseases in Greenland Inuits (10). Among the many reported effects of n-3 EFA, a prominent one is the hypotriglyceridemic effect of eicosapentaenoic acid (EPA), the precursor of 3-series eicosanoids (11). Supplementation with EPA was reported, on the other hand, to depress DGLA levels in plasma phospholipids relative to baseline values (12), which could lead to a decrease in PGE_1 synthesis. In order to prevent this DGLA depression, combined treatment with evening primrose oil and fish oil containing GLA and EPA, respectively, has been examined and resulted in the increase of DGLA (13) or in the ratio of DGLA to AA in rats (14). In the present paper, we examined the dietary effect of 1,3-biseicosapentaenoyl-2-γ-linolenoyl glycerol [symmetrical triacylglycerol (STG)] on fatty acid composition of guinea pigs, which have Δ5-desaturase activity (from DGLA to AA) similar to that of humans (15).

EXPERIMENTAL PROCEDURES

Materials. An STG shown in Figure l was chemically synthesized from EPA, GLA, and dihydroxyacetone (16). EPA (and EPA ethyl ester: EPA-E), GLA and soybean oil (SBO) were purchased from Nippon Chemical Feed Co. Ltd. (Hakodate, Japan) Idemitsu Materials Co. Ltd. (Okayama, Japan), and Sanwa Yushi Kogyo Co. Ltd. (Tokyo, Japan), respectively. The purities of EPA, EPA-E, GLA, and STG used in the present study were confirmed to be over 95%. A 30% lipid emulsion (STG, EPA-E, and SBO) was prepared by mixing 1.5 mL lipid and 3.5 mL 0.9% NaCl containing 0.05 g arabic gum and

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; AveCN, average carbon number of all the esterified fatty acids; DGLA, dihomo-γ-linolenic acid; EFA, essential fatty acid; EPA, eicosapentaenoic acid; EPA-E, eicosapentaenoic acid ethyl ester; GLA, γ-linolenic acid; LA, linoleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SBO; soybean oil; STG, symmetrical triacylglycerol 1,3-biseicosapentaenoyl-2-γ-linolenoyl glycerol; TG, triacylglycerol; TL, total lipids; total n-3, mol percentage of total n-3 EFA; total n-6, mol percentage of total n-6 EFA; UI, unsaturation index.

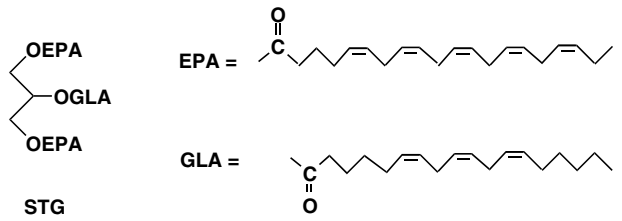

FIG. 1. Structural formula of a symmetrical triacylglycerol (STG).

0.05 g dL-α-tocopherol by ultrasonication with cooling on ice [similar to the method of Nilsson *et al.* (17)].

Animals. Five-week-old male Hartley strain guinea pigs (SLC, Japan Inc., Japan) were used. They were housed in a room maintained at a constant temperature (25°C) and kept on a 12:12 h light–dark cycle. All animals had free access to a standard pellet diet (RC-4; Oriental Yeast Co. Ltd., Tokyo, Japan) and water. The lipid emulsions were given by gavage once a day for 14 d. The amount of the lipid emulsion was adjusted to 1000 mg lipid/kg body weight/d. The guinea pigs were divided into four groups: STG ($n = 14$), EPA-E ($n = 12$), SBO ($n = 7$), and control with the standard diet only ($n = 10$).

Analysis of fatty acid composition. After 14 d of treatment, the animals were anesthetized by an injection of sodium pentobarbital into the femoral muscle (100 mg/kg). A common carotid artery was canulated to monitor arterial blood pressure (18). Heparinized blood (1% heparin, 1000 I.U.; Novo Industry, Bagsvaerd, Denmark) was collected from the carotid artery, and plasma was separated by centrifugation ($1000 \times g$, 10 min) and stored at −20°C until further analysis. After a catheter was inserted into the portal vein, the liver was perfused with saline solution to wash out blood, excised, and also stored at −20°C until further analysis. Plasma total lipids (TL) were measured by Hatch and Lees' method (19). A portion of the liver tissue was added to an ice-cooled solution of chloroform/methanol (1:2, vol/vol) and homogenized using an Ace Homogenizer AM-7 (Nihonnseiki-Seisakusho Ltd., Tokyo, Japan) at 10,000 rpm for 2 min. TL from the plasma and the homogenized liver were extracted by Bligh and Dyer's method (20). The extracted lipids from the liver were further separated by thin-layer chromatography (silica gel 60, 20 × 20 cm, 0.5 mm; Merck, Darmstadt, Germany) using chloroform/methanol/water (65:25:4, vol/vol/vol) as a developing solvent. The separated triacylglycerol (TG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) fractions were scraped and their fatty acid compositions determined. The fatty acids of these fractions were converted to their methyl esters using methanolic boron trifluoride after Metcalfe and Schmitz (21). The esters obtained were analyzed by gas–liquid chromatography using a Shimadzu GC-8A with a glass capillary column (SGE BPX-70; 0.25 mm × 25 m; Tokyo, Japan) at 200°C using nitrogen as a carrier gas (1.0 kg/cm^3). They were identified by the retention time of fatty acid methyl ester standards (Nu-Chek-Prep, Inc., Elysian, MN).

Statistical analysis. The results were expressed as mean ± standard error. Statistical significance was determined by one-way analysis of variance followed by post-hoc Duncan test.

RESULTS

There were no differences in weight or arterial blood pressure among four dietary groups, though the group fed the standard diet showed a slightly higher body weight at the end of the experimental periods. Thus, the four experimental groups with or without gavage of the lipid emulsions were comparable. Daily average amounts of the standard diet, whose fatty acid composition was close to that of SBO, given to the animals of four groups were in the range from 70 to 100 g/kg, or from 2.0 to 2.9 g lipid/kg. It followed that the three experimental groups were given additionally 1 g lipid/kg, i.e., from a half to one-third of the amount of lipid obtained from the standard diet.

The levels of cholesterol, TG, and phospholipid (PL) in plasma are shown in Table 1. The level of TG in the standard diet group was less than half of that in the SBO group in spite of similar fatty acid compositions in the diets of the two groups. This might be the effect of a lower consumption of dietary TG without gavage of lipid emulsion. Therefore, we omitted the standard diet group in the following comparison of fatty acid composition in tissue lipids. Compared with the SBO group, the EPA-E group had significant decreases in all the levels of cholesterol, TG, and PL ($P < 0.01$), indicative of a strong hypolipidemic effect of EPA-E. In the STG group, on the other hand, only the TG level was significantly decreased ($P < 0.05$).

The fatty acid compositions of plasma TL are shown in Table 2. In the EPA-E group, the levels of EPA and 22:5n-3 increased significantly compared with those of the SBO group ($P < 0.01$). As a result, the EPA/AA ratio in EPA-E group was more than 25 times that in the SBO group. The ratio of DGLA/AA in the EPA-E group was less than half that in the SBO group. In the STG group, on the other hand, both the levels of n-6 EFA (i.e., GLA, DGLA, and AA), and n-3 EFA (i.e., EPA and 22:5 n-3) were increased significantly compared with those of the SBO group ($P < 0.01$ except AA and $P < 0.05$ for AA). Increase in the AA level was less than those of GLA and DGLA. The ratio of DGLA/AA in the STG group was 0.367, i.e., 3.5 times that of the SBO group; the

TABLE 1
Plasma Cholesterol, Triacylglycerol, and Phospholipid[a]

	Diet only ($n = 10$)	SBO ($n = 7$)	STG ($n = 14$)	EPA-E ($n = 12$)
CHOL (mg/dL)	38.8 ± 2.5	36.3 ± 4.2	33.5 ± 2.5	27.3 ± 2.1**
TG (mg/dL)	35.7 ± 7.6**	76.3 ± 8.8	45.8 ± 5.9*	38.8 ± 6.6**
PL (mg/dL)	34.2 ± 1.7	31.3 ± 3.6	29.4 ± 1.6	25.4 ± 1.7**

[a]CHOL, cholesterols; TG, triacylglycerols; PL, phospholipids STG, symmetrical triacylglycerol, 1,3-biseicosapentaenoyl-2-γ-linolenoylglycerol; EPA-E, eicosapentaenoic acid ethyl ester.
*Significantly different from soybean oil (SBO) at $P < 0.05$; **$P < 0.01$.

TABLE 2
Fatty Acid Composition of Plasma Total Lipid of STG, EPA-E, and SBO Groups[a]

	STG (n = 14)	EPA-E (n = 12)	SBO (n = 7)
16:0	21.98 ± 1.32	22.80 ± 0.87	21.70 ± 1.84
18:0	11.04 ± 0.73	10.77 ± 0.55	8.57 ± 1.18
18:1n-9	12.31 ± 0.81	13.38 ± 0.63	13.84 ± 1.08
18:2n-6	23.86 ± 1.11*	21.85 ± 0.89**	31.14 ± 2.61
18:3n-6	1.77 ± 0.25**	0.01 ± 0.01	0.10 ± 0.05
18:3n-3	7.50 ± 0.41	7.12 ± 0.59	8.04 ± 1.12
20:3n-6	0.79 ± 0.15**	0.07 ± 0.04	0.13 ± 0.04
20:4n-6	2.16 ± 0.25*	1.40 ± 0.22	1.24 ± 0.17
20:5n-3	4.24 ± 0.30**	4.06 ± 0.51**	0.13 ± 0.04
22:5n-3	1.39 ± 0.21**	1.26 ± 0.20**	0.22 ± 0.09
22:6n-3	0.28 ± 0.08	0.17 ± 0.06	0.21 ± 0.01
EPA/AA	1.965	2.908	0.106
DGLA/AA	0.367	0.050	0.104
Total n-6	28.59%	23.33%	32.61%
Total n-3	13.40%	12.62%	8.60%
n-3/n-6	0.469	0.541	0.264
AveCN	15.5	14.6	15.0
UI	1.29	1.12	1.09

[a]Values are in mol%, mean ± SE. *Significantly different from SBO at $P < 0.05$; **$P < 0.01$. Abbreviations: total n-6, mol% of total n-6 essential fatty acid (EFA); Total n-3, mol% of total n-3 EFA; AveCN, average carbon number of all the esterified fatty acids; UI, unsaturation index; EPA/AA, eicosapentaenoic acid/arachidonic acid. DGLA/AA, dihomo-γ-linolenic acid/AA. See Table 1 for other abbreviations.

TABLE 3
Fatty Acid Composition of Liver Phosphatidylcholine of STG, EPA-E, and SBO Groups[a]

	STG (n = 14)	EPA-E (n = 12)	SBO (n = 7)
16:0	16.53 ± 0.57	15.11 ± 0.65*	16.84 ± 0.27
18:0	30.78 ± 1.01**	35.85 ± 1.81**	25.13 ± 1.69
18:1n-9	6.79 ± 0.55	8.29 ± 0.33	7.68 ± 0.57
18:2n-6	24.08 ± 0.98**	25.47 ± 1.27**	35.98 ± 2.15
18:3n-6	1.49 ± 0.14**	0.26 ± 0.09	0.16 ± 0.05
18:3n-3	1.25 ± 0.45	0.76 ± 0.22	1.28 ± 0.25
20:3n-6	2.39 ± 0.23**	0.63 ± 0.09	0.51 ± 0.04
20:4n-6	3.48 ± 0.26	3.62 ± 0.41	2.71 ± 0.27
20:5n-3	1.70 ± 0.37**	1.72 ± 0.23**	0.20 ± 0.03
22:5n-3	1.29 ± 0.12**	1.52 ± 0.21**	0.32 ± 0.04
22:6n-3	0.56 ± 0.09	0.30 ± 0.05*	0.46 ± 0.06
EPA/AA	0.488	0.475	0.076
DGLA/AA	0.687	0.173	0.187
Total n-6	31.44%	29.98%	39.35%
Total n-3	4.80%	4.29%	2.27%
n-3/n-6	0.153	0.143	0.058
AveCN	16.2	16.7	16.2
UI	1.03	0.97	1.02

[a]Values are in mol%, mean ± SE. *Significantly different from SBO at $P < 0.05$; **$P < 0.01$. See Tables 1 and 2 for abbreviations..

EPA/AA ratio in the STG group was 18 times that of the SBO group.

The average carbon number of all the esterified fatty acids (AveCN in Table 2) was 15.5, longer than those in the other two groups. The unsaturation index (UI in Table 2), the average number of double bonds in each fatty acid molecule, of the STG group was 1.29, also higher than those of the other two groups.

The fatty acid composition of the liver lipids is shown in Tables 3–5. In PC (Table 3), the levels of GLA, DGLA; EPA, 22:5n-3; and 18:0 were increased in the STG group and the levels of EPA, 22:5n-3, and 18:0 were increased in the EPA-E group. However, the AA levels did not differ significantly among the three groups. As a result, the DGLA/AA ratio of the STG group was 0.687, i.e., 3.5 times as great as those of the SBO and EPA-E groups. At the same time, the EPA/AA ratios of the STG and EPA-E groups were more than 6 times that of the SBO group. Levels of both n-6 and n-3 EFA were largest in the STG group.

In PE (Table 4), the levels of AA as well as α-linolenic acid (ALA), 18:3n-3, were significantly decreased in both the STG and EPA-E groups compared with the SBO group. As reported (13), EPA seems to exert an inhibitory effect on the conversion of DGLA to AA, resulting in a lower level of AA in the two groups. Consequently, the DGLA/AA ratio of the STG group was 0.237, i.e., 6 times those of the other groups, a greater effect in PE than PC. The content of n-3 EFA in PE was about 14%, three times those in PC in both the STG and EPA-E groups. As a result, the EPA/AA ratios of the STG and EPA-E groups were 13 and 16 times, respectively, that of the SBO group.

Contrary to the results of PC and PE in which LA levels decreased in the STG and EPA-E groups, the LA levels in TG (Table 5) did not differ from that in the SBO group. The ratios of DGLA/AA and EPA/AA of the STG group were 11 and 3.8 times, respectively, those of the SBO group. The level of DGLA/AA in the EPA-E group was higher than that of the SBO group only in the liver TG fraction. The levels of UI and both n-6 and n-3 EFA were largest in the STG group.

TABLE 4
Fatty Acid Composition of Liver Phosphatidylethanolamine of STG, EPA-E, and SBO Groups[a]

	STG (n = 14)	EPA-E (n = 12)	SBO (n = 7)
16:0	8.33 ± 0.24	9.52 ± 0.54*	8.09 ± 0.34
18:0	34.59 ± 0.75*	35.83 ± 1.35*	30.34 ± 1.53
18:1n-9	3.88 ± 0.39	4.34 ± 0.28	3.60 ± 0.36
18:2n-6	20.35 ± 0.80**	22.20 ± 1.60**	35.22 ± 0.95
18:3n-6	0.94 ± 0.06**	0.00 ± 0.00	0.26 ± 0.06
18:3n-3	1.26 ± 0.32*	1.26 ± 0.18*	2.06 ± 0.26
20:3n-6	1.78 ± 0.14**	0.23 ± 0.06	0.35 ± 0.07
20:4n-6	7.53 ± 0.32**	6.32 ± 0.42**	9.08 ± 0.49
20:5n-3	5.67 ± 0.57**	5.74 ± 0.49**	0.51 ± 0.04
22:5n-3	4.92 ± 0.38**	4.71 ± 0.54**	1.09 ± 0.04
22:6n-3	2.14 ± 0.17	2.34 ± 0.19	1.90 ± 0.15
EPA/AA	0.752	0.91	0.056
DGLA/AA	0.237	0.04	0.039
Total n-6	30.61%	28.75%	44.90%
Total n-3	13.99%	14.05%	5.56%
n-3/n-6	0.457	0.489	0.124
AveCN	16.9	17.0	16.8
UI	1.52	1.45	1.38

[a]Values are in mol%, mean ± SE. *Significantly different from SBO at $P < 0.05$; **$P < 0.01$. See Tables 1 and 2 for abbreviations..

TABLE 5
Fatty Acid Composition of Liver Triacylglycerol of STG, EPA-E, and SBO Groups[a]

	STG (n = 14)	EPA-E (n = 12)	SBO (n = 7)
16:0	27.77 ± 0.98	29.43 ± 1.07	28.44 ± 1.47
18:0	4.55 ± 0.36	4.23 ± 0.42	3.94 ± 0.21
18:1n-9	16.81 ± 0.27**	19.48 ± 0.54	19.08 ± 0.76
18:2n-6	24.98 ± 0.91	23.90 ± 0.83	23.78 ± 0.60
18:3n-6	1.45 ± 0.11**	0.15 ± 0.09	0.18 ± 0.02
18:3n-3	9.22 ± 0.41	8.73 ± 0.58	8.02 ± 0.66
20:3n-6	0.62 ± 0.17**	0.12 ± 0.05	0.10 ± 0.03
20:4n-6	0.67 ± 0.06*	0.34 ± 0.06	0.42 ± 0.07
20:5n-3	2.25 ± 0.16**	2.15 ± 0.33**	0.13 ± 0.03
22:5n-3	1.04 ± 0.11**	0.80 ± 0.11**	0.21 ± 0.02
22:6n-3	0.19 ± 0.04	0.09 ± 0.03	0.12 ± 0.02
EPA/AA	3.382	6.232	0.309
DGLA/AA	0.927	0.347	0.243
Total n-6	27.71%	24.51%	24.47%
Total n-3	12.69%	11.77%	8.48%
n-3/n-6	0.458	0.480	0.347
AveCN	15.7	15.6	14.7
UI	1.21	1.11	0.96

[a]Values are in mol%, mean ± SE. *Significantly different from SBO at $P < 0.05$; **$P < 0.01$. See Tables 1 and 2 for abbreviations..

DISCUSSION

There have been several studies on the dietary effect of combination of GLA and EPA in the form of TG mixtures using mainly evening primrose oil and fish oil, but there has been no previous study in the form of a synthetic, mixed TG. The combination of primrose oil and fish oil has been shown to have the following effects: inhibition of bone resorption (22), decrease in malignancy of mammary carcinomas of rats (23,24), favorable reduction of plasma cholesterol and TG levels in the nephrotic syndrome (25), modulation of the renal syndrome induced by cyclosporine (26), alleviation of gastric hemorrhage caused by aspirin (27), reduction of intrarenal calcification (28), amelioration of radiation damage to the skin (29), and amelioration of lung injury induced by endotoxin (30). Decreased *ex vivo* conversion from AA to T_XB_2 was observed in human platelets after the administration of both GLA and EPA in spite of increased AA content of the platelets (31).

In terms of effect on plasma lipids, EPA-E had greater hypolipidemic properties than STG. On the other hand, in terms of EPA incorporation, EPA content in liver lipid was almost the same in the STG and EPA-E groups, and EPA content in plasma TL was somewhat higher (though not significantly) in the STG group than in the EPA-E group. Considering that the amount of EPA administered in the EPA-E group was almost 1.5 times that of the STG group, EPA may be absorbed more effectively as the glycerol ester than as the ethyl ester in guinea pigs. Higher absorption of EPA glycerol than EPA-E was reported in the human (32,33), but the opposite results have been reported in rats (34). In all the tissue lipids, the STG group had higher UI than the EPA-E group even though there is a lower UI in the STG diet than in the EPA-E diet. These results suggest that greater amounts of desaturase products as a whole were synthesized in the STG groups than in the other two groups.

Crozier *et al.* (35) reported the effect of feeding black currant seed oil containing GLA (17.1 mol%) and ALA (12.7 mol%) to guinea pigs. The levels of GLA and DGLA in liver lipids increased but the levels of AA remained relatively stable, which was similar to the results of the present report, indicative of a low activity of Δ5-desaturase in these animals (15,36). Δ5-Desaturase activity was reportedly found in guinea pig megakaryocytes but not platelets (37). The ratios of DGLA/AA in liver PL with black currant oil and the STG can be compared, though GLA content of the diets and the way of dieting were not the same. The content of GLA in the black currant oil diet was 17.1 mol% (35) and that in the STG diet was estimated at about 10 mol%. This estimation was based on the amount of STG administered (1 g/kg/d at 33.3 mol% GLA) and the lipid from the standard diet (2–2.9 g/kg/d at 0% GLA), which gave 11.1–8.5 mol%. The ratios of DGLA/AA in PC and PE with black currant oil were 0.40 and 0.15, respectively, and 0.687 (PC, Table 3) and 0.237 (PE, Table 4) in the STG group. The apparently weaker effect of black currant oil on the increase in the ratio of DGLA/AA seems to be attributable to the higher GLA distribution in the 3-position of TG (38), indicating that TG molecules with GLA at the 2-position are more effective in increasing the level of n-6 EFA, just as 2-positioned EPA molecules are (32). Moreover, EPA in STG seems to inhibit the conversion of DGLA to AA as reported with EPA in fish oil (13). The ratio of DGLA/AA in the STG group obtained in the present study was eventually higher than when feeding black currant oil, in spite of lower GLA content.

Miller and Zibon (39) reported that the DGLA/AA ratio in epidermal PL of guinea pigs being fed borage oil (GLA: 25.2%) was 3 times larger than those fed safflower oil. Moreover, guinea pig epidermal homogenate was reported to form PGE_1 and 15-hydroxy-8,11,13-eicosatrienoic acid (9), the latter of which has been suggested to be an inhibitor of 5- and 12-lipoxygenases (40). High levels of these ratios may be responsible for antithrombotic effect of STG reported elsewhere (41).

The level of docosahexaenoic acid, 22:6n-3, in plasma and liver lipids remained relatively constant among the three groups suggesting that Δ4-desaturase activity or other pathways for increasing docosahexaenoic acid synthesis were very low in guinea pigs (15).

In summary, in terms of the ratios of DGLA/AA and EPA/AA, the STG group had a more favorable fatty acid profile than the EPA-E and SBO groups both in plasma and liver lipids. The ratios of DGLA/AA and EPA/AA in the STG group were 0.687 and 0.488 (in PC) and 0.237 and 0.752 (in PE), respectively. The ratio of DGLA/AA obtained in the present study with the STG diet, the highest so far reported in guinea pigs, as well as the high EPA/AA ratio may lead to physiological changes, including increased production of 1- and 3-series eicosanoids.

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Distribution of Cholesterol Sulfate and Its Anabolic and Catabolic Enzymes in Various Rabbit Tissues

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ABSTRACT: Cholesterol sulfate (CS) recently has been shown to be involved in signal transduction pathway. To evaluate its functional significance, we determined the concentration of CS, and the specific activities of cholesterol sulfotransferase and CS sulfatase in various tissues of rabbit, and compared them with the concentration of sulfoglycolipids in rabbit tissues. CS was present in the epithelia and mucosa, but not in the tunica muscularis, of the digestive tract, trachea, uterine endometrium and uterine cervix. It was also present in lung, spleen, kidney, prostate, skin, hair, and nail at relatively high concentrations. Its concentration in the uterine endometrium was nine times higher in pseudopregnant rabbits than in nonpregnant rabbits because of activation of cholesterol sulfotransferase and inhibition of CS sulfatase in the pseudopregnant rabbits. Sulfoglycolipids were not detected in the uterine endometria of either nonpregnant- or pseudopregnant rabbits. However, sulfoglycolipids were detected at relatively high concentrations in the cerebrum, cerebellum, stomach, duodenum, jejunum, testis, and kidney of rabbits and thus the tissues in which both sulfolipids were detected were the gastrointestinal tract and kidney. In the digestive tract, the concentration of CS decreased in the order esophagus, stomach, duodenum, and jejunum, but that of sulfatide increased in the same order, indicating distribution of CS in the squamous epithelium. In addition, both CS and sulfatide were detected in the serum. On the other hand, CS sulfatase activity was detected in all tissues examined, even in hair, from which the enzyme was liberated by brief sonication, and its highest specific activity was detected in the liver. The specific activity of cholesterol sulfotransferase varied among the tissues examined and was found to be significantly high in the esophageal epithelium and the uterine endometrium of pseudopregnant rabbit, indicating involvement of cholesterol sulfation in the formation of epithelium.

Lipids 32, 599–604 (1997).

Membrane constituents are known to change in composition and concentration in association with cellular proliferation and differentiation. In a previous study (1), we found that cholesterol sulfate (CS) was characteristically expressed in the uterine endometria of rabbits induced into pseudopregnancy by the administration of 17β-estradiol and chorionic gonadotropin. This suggested the involvement of CS in the process of implantation of a fertilized egg in the uterine endometria of pseudopregnant rabbits. In addition, CS was shown to be a major constituent of mammalian skin (2), in which synthesis occurred in the epidermal spinous layer (3), and the highest concentration was observed in the stratum granulosum (4), where it is important for normal desquamation (5–7). Its expression in keratinocytes was found to be a differentiation-associated phenomenon (8), and it exhibits an ability to activate protein kinase C-isoenzymes, eta, epsilon and zeta (9,10), suggesting its involvement in the multistep process of differentiation of keratinocytes. Although knowledge has been accumulated of the functional significance of CS, particularly in the epidermis, information on its distribution in various tissues was limited (11), which led us to examine its concentration in various tissues of rabbit. Also, we determined the specific activities of cholesterol sulfotransferase and CS sulfatase and compared them with the concentrations of sulfoglycolipids by thin-layer chromatography (TLC)-immunostaining with a newly established antisulfatide monoclonal antibody and TLC-densitometry.

MATERIALS AND METHODS

Preparation of tissues. Rabbits (New Zealand white, 6 mon old, virgin female) were purchased from Japanese Biological Materials Co. (Tokyo, Japan). Each rabbit was injected intramuscularly with 0.5 mg 17β-estradiol on the first day. After 72 h, 250 IU of human chorionic gonadotropin (Sigma, St. Louis, MO) in 0.5 mL saline solution was injected intravenously. The rabbit was sacrificed 4 d after the second injection by means of an intravenous injection of pentobarbital sodium. Various tissues were removed and washed with phosphate-buffered saline (PBS). The epithelial layer of the esophagus, stomach, duodenum, jejunum, and trachea and the uterine endometrium were scraped from the tunica muscularis. The tissues were homogenized in water with a Polytron homogenizer (Kinematica, Luzern, Switzerland) and then lyophilized for the quantitative analyses of lipids. A part of various tissues, including hair, was homogenized in 5 vol of 0.25 M sucrose with a Polytron homogenizer and an ultrasonicator and then centrifuged at 700 × *g* for 10 min to remove

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Abbreviations: BSA, bovine serum albumin; CS, cholesterol sulfate; DG, 1-alkyl-2-acylglycerol; MG, 1-alkyl glycerol; NFA, nonhydroxy fatty acid; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

cell debris, and the resultant supernatant was used for the determination of cholesterol sulfotransferase and CS sulfatase. Protein was measured by the dye-binding method with bovine serum albumin (BSA) as the standard (12).

Preparation of lipids. Total lipids were extracted from the lyophilized powder of various tissues of rabbit with chloroform/methanol/water (20:10:1, 10:20:1, and 10:10:1, by vol), and the combined extracts were applied onto a column packed with DEAE-Sephadex A-25 (acetate form; Pharmacia, Uppsala, Sweden). After elution of the unabsorbed lipids with 3 vol of chloroform/methanol (1:1, vol/vol) and 1 vol of methanol, the acidic lipids absorbed on the column were eluted with 10 vol of 0.3 M sodium acetate in methanol, and then saponified with 0.5 M sodium hydroxide in methanol at 40°C for 40 min to cleave the ester-containing acidic phospholipids (13). After removal of salts by dialysis, the solution was evaporated to dryness and the acidic lipids obtained were dissolved in chloroform/methanol (1:1, vol/vol).

Assay of cholesterol sulfotransferase. Cholesterol sulfotransferase was measured with the supernatant obtained after centrifugation at $700 \times g$ for 10 min of the homogenates of various tissues of rabbit. The assay mixture comprised 0.2 mM cholesterol, 0.5 mM dithiothreitol, 1.35 μM ^{35}S-phosphoadenosine phosphosulfate (74.0 GBq/mmol; New England Nuclear, Boston, MA), 100 mM phosphate buffer, pH 7.3, 10 mg/mL 2-hydroxypropyl-β-cyclodextrin, and 50 μg enzyme protein in a final volume of 100 μL. After incubation at 37°C for 1 h, the reaction was terminated by the addition of 2 mL of chloroform/methanol (2:1, vol/vol) containing 0.4 μg of CS and 0.5 mL of H_2O. Then the solution was mixed well and centrifuged at $1500 \times g$ for 2 min. The upper phase was removed, and the lower phase was air-dried. The constituents of the residue were separated by TLC with chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, by vol) as the developing solvent. The radioactivity of the band corresponding to CS was counted with a BAS-2000 image analyzer (Fuji Film, Tokyo, Japan).

Assay of CS sulfatase. CS sulfatase was measured with the same supernatants as those used for cholesterol sulfotransferase. The assay mixture comprised 11.0 μM ^{3}H-1,2,6,7-CS, 50 mM imidazole-HCl, pH 7.0, 50 μg Triton X-100, and 50 μg enzyme protein in a final volume of 100 μL. After incubation at 37°C for 1 h, the reaction was terminated by the addition of 1.5 mL of chloroform/methanol (2:1, vol/vol) containing 0.2 μg of cholesterol and 0.5 mL of water. The solution was mixed well and then centrifuged at $700 \times g$ for 2 min. The upper phase was removed, and the lower phase was air-dried. The constituents of the residue were separated by TLC with chloroform/methanol/acetic acid (100:2:1, by vol) as the developing solvent. The band corresponding to that of cholesterol was cut out to count the radioactivity with a liquid scintillation counter (Liquid Scintillation Analyzer TRI-CARB 1500; Packard Co., Tokyo, Japan).

Monoclonal antibody. A murine monoclonal antisulfatide antibody, TCS-1, was prepared by immunizing C57BL mice with sulfatide purified from human brain, hybridizing splenocytes with myeloma cells (P3.X63.AG8.653), and then monitoring the antibody-producing hybridomas by enzyme-linked immunosorbent assay with sulfatide as the antigen.

TLC and TLC-immunostaining. Acidic lipids were chromatographed on a high-performance TLC plate (E. Merck Co., Darmstadt, Germany) with the solvent system of chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol), and the bands were detected with cupric acetate–phosphoric acid for CS, orcinol–H_2SO_4 for sulfoglycolipids, resorcinol–HCl for sialoglycolipids, and ferric chloride–H_2SO_4 for steroids. The standards for quantitative determination by TLC were chemically synthesized CS, I^3SO_3-GalCer for sulfoglycolipids and II^3NeuAcα-LacCer for sialoglycolipids. The density of the spots was determined at a sample wavelength of 420 nm and a control wavelength of 700 nm with a TLC-densitometer (CS-9000; Shimadzu, Kyoto, Japan). The lower limit for detection was 0.1 μg, and the standard curves were linear up to 1.5 μg. For TLC-immunostaining of sulfolipids, a plastic-coated TLC plate (Sigma-Aldrich) was used for the development of acidic lipids with chloroform/methanol/water (65:35:8, by vol). After blocking the plate with 1% bovine serum albumin (BSA) in PBS at 4°C overnight, it was incubated with the murine monoclonal antisulfatide antibody diluted 1:500 with 1% BSA in PBS (wt/vol) at 37°C for 2 h, and was washed five times with 0.1% Tween-20 in PBS (wt/vol). The antibody remaining on the plate was detected with peroxidase-conjugated antimouse-IgG+M antiserum (Capple Lab., Cochranville, PA) diluted 1:500 with 1% BSA in PBS at 37°C for 1 h and enzyme substrate (H_2O_2 and 4-chloro-1-naphthol). The density of the spots was determined with a TLC-densitometer as described above.

RESULTS

Characterization of the monoclonal antisulfatide antibody. The epitope structure required for binding the monoclonal anti-I^3SO_3-GalCer antibody (TCS-1) was essentially identical with that reported by Fredman *et al.* (14), reacting with glycolipids having the galactose substituted at the 3-position with a sulfate group, I^3SO_3-GalCer, II^3SO_3-LacCer and I^3SO_3-GalDG (seminolipid), but not with II^3SO_3-Gg_3Cer or CS (Fig. 1). The lack of reactivity of I^3SO_3-GalMG with anti-I^3SO_3-GalCer antibody indicated that the structure of the hydrophobic side chain affected the reactivity of the carbohydrate epitope on the TLC plate. In fact, I^3SO_3-GalCer with 2-hydroxy fatty acid-sphingosine or nonhydroxy fatty acid (NFA)-phytosphingosine was more reactive than I^3SO_3-GalCer with NFA-sphingosine. And, although the reactivity of I^3SO_3-GalDG was similar to that of I^3SO_3-GalCer with NFA-sphingosine, that of II^3SO_3-LacCer was twice that of I^3SO_3-GalCer with NFA-sphingosine (Fig. 2).

Distribution of CS in various tissues of rabbit. On TLC of the acidic lipid fraction, corresponding to 2 mg of protein (Fig. 3), CS was found to be present in relatively high concentration in various tissues, other than heart, liver, adrenal gland, cerebrum, cerebellum and testis, of rabbit. Its structure

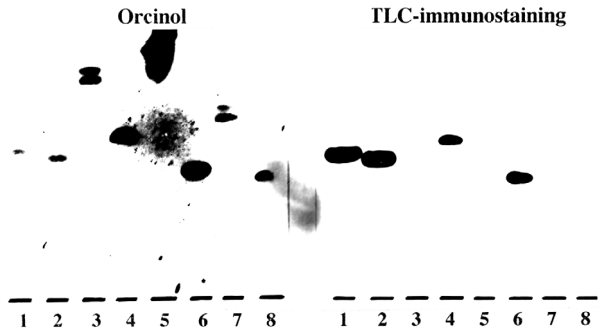

FIG. 1. Immunostaining characterization of the monoclonal anti-I^3SO_3-GalCer antibody (TCS-1). Standard glycolipids (0.5–1.5 μg), purified in our laboratory, were developed on a plastic-coated silia gel plate with chloroform/methanol/water (65:35:8, by vol), and then visualized with orcinol-H_2SO_4 reagent or monoclonal antibody TCS-1. 1, I^3SO_3-GalCer(NFA); 2, I^3SO_3-GalCer(HFA); 3, GalCer(NFA + HFA); 4, I^3SO_3-GalDG; 5, GalDG; 6, II^3SO_3-LacCer; 7, LacCer; 8, I^3SO_3-GalMG.

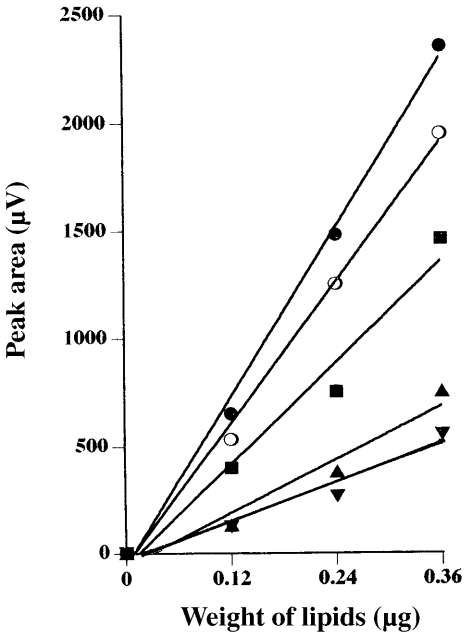

FIG. 2. Reactivities of several sulfoglycolipids with the monoclonal anti-I^3SO_3-GalCer antibody (TCS-1), as determined on TLC-immunostaining. Known amounts of several sulfoglycolipids were chromatographed with chloroform/methanol/water (65:35:8, by vol.), and then visualized with monoclonal antibody, TCS-1. The density of spots was determined with a TLC-densitometer as described in the text. ●, I^3SO_3-GalCer with HFA-sphingosine; ○, I^3SO_3-GalCer with NFA-phytosphingosine; ■, II^3SO_3-LacCer with NFA-sphingosine; ▲, I^3SO_3-GalCer with NFA-sphingosine; ▼, I^3SO_3-GalDG.

was confirmed by comparing its position on TLC with that of the standard CS, staining with ferric chloride reagent for steroid, and analyzing the materials from skin and uterine endometrium by negative ion fast atom bombardment mass spectrometry, which gave the ions characteristic for CS, $[M - H]^-$ at *m/z* 465, and sulfate-derived ions at *m/z* 80 and 97 (15). A relatively high concentration of CS (Table 1) was observed in the epithelia of the esophagus, uterine cervix and trachea, the mucosa of the gastrointestinal tract, the endometrium of the uterus from pseudopregnant rabbits, kidney, lung, skin, prostate, hair and nail. CS constituted 100 and 98.6% of the total acidic lipids in hair and nail, amounting to 2.3 and 1.4 nmol/g dry weight, respectively. Also, as reported previously (15), the concentration of CS in the uterine endometrium of pseudopregnant rabbits was confirmed to increase to 8.75 times higher than that in nonpregnant rabbits. In addition, CS was in the serum at a concentration of 0.1 nmol/mg protein, which was similar to that of I^3SO_3-GalCer in the serum. On the other hand, sulfoglycolipids showed a more restricted tissue distribution than that of CS. As shown in Figures 3 and 4, I^3SO_3-GalCer detected on TLC-immunostaining with anti-I^3SO_3-GalCer antibody was present in the mucosa of stomach, duodenum and jejunum, kidney, cerebrum, cerebellum, lung, and serum. Interestingly, the molar percentage of CS in the total sulfolipids in the digestive tract decreased in the order esophageal epithelium (100.0%), gastric mucosa (91.9%), duodenal mucosa (54.1%), and jejunal

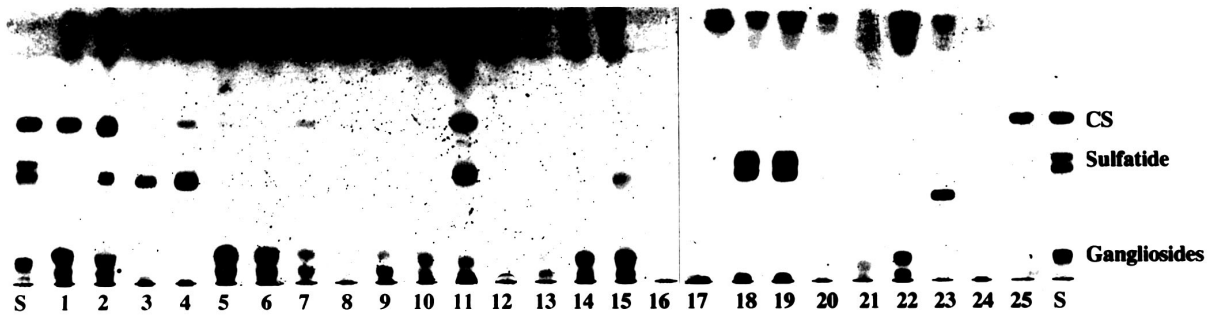

FIG. 3. TLC of acidic lipids from various tissues of rabbit. The developing solvent was chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, by vol), and the spots were visualized with cupric acetate-phosphoric acid reagent. 1, esophageal epithelium; 2, gastric mucosa; 3, duodenal mucosa; 4, jejunal mucosa; 5, tracheal epithelium; 6, uterine endometrium (nonpregnancy); 7, uterine endometrium (pseudopregnancy); 8, skin; 9, uterine cervical epithelium; 10, lung; 11, kidney; 12, heart; 13, liver; 14, spleen; 15, adrenal gland (female); 16, ovary; 17, thymus; 18, cerebrum; 19, cerebellum; 20, serum; 21, hair; 22, adrenal gland (male); 23, testis; 24, prostate; 25, nail; S, standard lipid mixture, CS, I^3SO_3-GalCer and II^3NeuAcα-LacCer.

TABLE 1
Concentrations of Cholesterol Sulfate and Sulfoglycolipids in Various Tissues of Rabbit

Tissues	Cholesterol sulfate (nmol/mg protein)	Sulfatide (nmol/mg protein)
Esophageal epithelium	2.55 ± 0.50	trace
Gastric mucosa	3.99 ± 1.89	0.35 ± 0.17
Duodenal mucosa	0.59 ± 0.22	0.50 ± 0.28
Jejunal mucosa	0.69 ± 0.26	1.31 ± 0.28
Tunica muscularis (esophagus, stomach, duodenum, Jejunum)	n.d.	n.d.
Tracheal epithelium	0.42 ± 0.03	0.12 ± 0.03
Lung	0.58 ± 0.27	trace
Kidney	2.75 ± 0.19	0.96 ± 0.29
Liver	trace	trace
Heart	n.d.	n.d.
Spleen	0.19 ± 0.14	trace
Serum	0.13 ± 0.03	0.13 ± 0.03
Cerebrum	trace	12.48 ± 0.83
Cerebellum	trace	9.88 ± 2.55
Skin[b]	10.23 ± 0.83	trace
Hair[b]	2.34 ± 0.79	n.d.
Nails[b]	1.44 ± 0.13	n.d.
Uterine endometrium (nonpregnancy)	0.08 ± 0.03	n.d.
Uterine endometrium (pseudopregnancy)	0.65 ± 0.17	n.d.
Uterine Cervical epithelium	0.12 ± 0.03	n.d.
Ovary	trace	trace
Thymus	trace	trace
Adrenal gland (female)	trace	trace
Adrenal gland (male)	trace	trace
Testis	trace	0.40 ± 0.12
Prostate	0.33 ± 0.03	trace

[a]Data are expressed as the mean of triplicate experiments. Tissues other than the uterine endometrium of pseudopregnant rabbits and the adrenal gland of male rabbits were obtained from nonpregnant female rabbits (6 mon old).
[b]nmol/g of dry weight; trace amount (<0.01 nmol/mg protein); n.d., not detected.

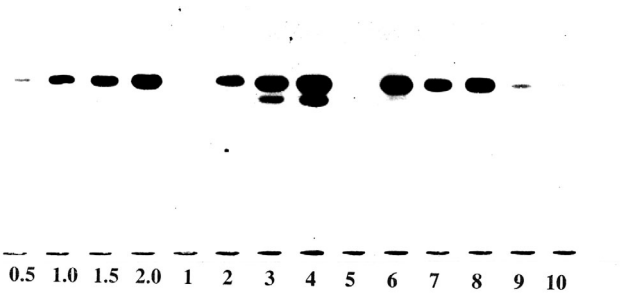

FIG. 4. TLC-immunostaining of acidic lipids from various tissues of rabbit. The developing solvent was the same as that in Figure 3, and the spots were located with the monoclonal anti-I^3SO_3-GalCer antibody. 0.5-2.0, μg of standard glycolipids, I^3SO_3-GalCer with NFA-sphingosine, purified in our laboratory. 1, esophageal mucosa; 2, gastric mucosa; 3, duodenal mucosa; 4, jejunal mucosa; 5, tracheal epithelium; 6, kidney; 7, cerebrum; 8, cerebellum; 9, serum; 10, lung.

mucosa (34.5%). Since the esophageal epithelium was composed of a laminated squamous epithelium, and the relative proportion of glandular epithelium increased in the order of gastric, duodenal and jejunal mucosas, in which I^3SO_3-GalCer was found to be highly concentrated in the apical and basal layers of glands on immunohistochemical staining with the anti-I^3SO_3-GalCer antibody (Cui Y. and Iwamori, M., to be published), CS was thought to be a component of the stratified and columnar epithelia in these digestive organs.

Specific activities of cholesterol sulfotransferase and CS sulfatase in various rabbit tissues. Relatively high specific activities of cholesterol sulfotransferase and CS sulfatase were observed in the esophageal epithelium of nonpregnant rabbits and the uterine endometrium of pseudopregnant rabbits, and in the liver, respectively (Table 2). The specific activity of cholesterol sulfotransferase in the uterine endometrium was 15 times higher in pseudopregnant rabbits than in nonpregnant rabbits, whereas that of CS sulfatase in pseudopregnant rabbits was one-third of that in nonpregnant rabbits. Thus, activation of cholesterol sulfotransferase and inhibition of CS sulfatase were found to contribute to the accumulation of CS in the endometrium of pseudopregnant rabbits. Although the specific activities of CS sulfatase in tissues other than liver were similar to each other, those of cholesterol sulfotransferase were quite different among the tissues examined and did not correspond with the concentration of CS. In addition, cholesterol sulfotransferase in the digestive tract and uterus was found to be present in the epithelial and endometrial layers but not in the tunica muscularis. Also, the specific activities of cholesterol sulfotransferase and dehydroepiandrosterone sulfotransferase in liver were 3.2 ± 0.7 pmol/mg protein/h and 1253.8 ± 15.0 pmol/mg protein/h, respectively, whereas those in the uterine endometrium of pseudopregnant rabbits were 1096.7 ± 283.7 pmol/mg protein/h and 1281.0 ± 89.4 pmol/mg protein/h, respectively, indicating that dehydroepiandrosterone sulfotransferase in liver is unable to catalyze the sulfation of cholesterol to the same level as that in the uterine endometrium. CS in the serum was thought to be derived through shedding from the tissues exhibiting synthetic activity for CS. Also, no synthetic activity was detected in hair exhibiting a high concentration of CS, which seemed to derive from the folliculus pili for the synthesis of hair, but the catalytic enzyme, CS sulfatase, was present in hair and was readily liberated by brief sonication, probably continuing the cleavage of CS in hair in the same manner as in the stratum corneum (5). Liver, testis, adrenal, ovary, cerebrum, and cerebellum tissues, in which CS was present in trace amounts, contained CS sulfatase, probably for processing of CS as a source of steroids.

DISCUSSION

CS has been characterized as a molecular marker for the epithelial differentiation of epidemal keratinocytes (8) as well as of tracheal epithelial cells (16), and has been proposed to act as an adhesive agent essential for the orderly adhesion-desquamation behavior of the epidermis (7). In accordance

TABLE 2
Specific Activities of Cholesterol Sulfotransferase and Cholesterol Sulfate Sulfatase in Various Tissues of Rabbit[a]

Tissues	Cholesterol sulfotransferase (pmol/mg protein/h)	Cholesterol sulfate sulfatase (pmol/mg protein/h)
Esophageal epithelium	1287.5 ± 522.9	8.4 ± 0.5
Gastric mucosa	10.0 ± 1.0	4.3 ± 0.4
Duodenal mucosa	2.6 ± 0.9	6.0 ± 0.5
Intestinal mucosa	84.3 ± 13.9	6.8 ± 0.7
Tracheal epithelium	119.9 ± 33.8	22.2 ± 1.3
Lung	0.4 ± 0.1	25.2 ± 2.3
Kidney	5.2 ± 1.4	6.7 ± 1.3
Liver	3.2 ± 0.7	254.3 ± 22.8
Heart	0.2 ± 0.1	6.6 ± 0.9
Spleen	0.8 ± 0.4	33.5 ± 4.0
Serum	0.0 ± 0.0	8.7 ± 0.5
Cerebrum	6.5 ± 1.2	18.4 ± 1.3
Cerebellum	4.7 ± 1.8	13.6 ± 0.8
Skin	40.4 ± 19.4	8.1 ± 0.5
Hair	0	26.8 ± 17.4
Uterine endometrium (nonpregnancy)	75.8 ± 21.7	27.9 ± 1.7
Uterine endometrium (pseudopregnancy)	1096.7 ± 283.7	9.5 ± 2.6
Uterine cervical epithelium	38.6 ± 8.7	24.9 ± 1.6
Ovary	9.2 ± 2.9	26.5 ± 2.0
Thymus	0.5 ± 0.3	25.0 ± 1.5
Adrenal gland (female)	0	27.2 ± 1.9
Adrenal gland (male)	0	15.2 ± 0.9
Testis	56.9 ± 4.7	34.3 ± 2.1
Prostate	184.5 ± 77.4	25.5 ± 1.7

[a]Data are expressed as the mean of triplicate experiments. Tissues other than the uterine endometrium of pseudopregnant rabbits and the adrenal gland of male rabbits were obtained from nonpregnant female rabbits (6 mon old).

with those findings, CS was localized in the epidermal layers, but not in the tunica muscularis of the digestive tract and uterus, and in the skin including hair and nail. In the uterine endometrium of pseudopregnant rabbits, a formation of the dendritic structure due to dilation of the epithelium was found to be associated with activation of cholesterol sulfotransferase and accumulation of CS. Apparently, the synthesis of CS in the endometrium was associated with the formation of epithelium and was regulated with steroids, in a similar manner to that of dehydroepiandrosterone sulfotransferase (17).

In the cases of primary cultures of tracheal epithelial cells and epidermal keratinocytes, activation of cholesterol sulfotransferase and accumulation of CS were observed in the confluent stage (8,16,18), and were enhanced by treatment with phorbol-12-myristate-13-acetate, tumor growth factor-β, and high Ca^{2+}, which are agents that induce squamous differentiation. The reasons why the specific activity of cholesterol sulfotransferase in various tissues was not correlated with the concentration of CS were thought to be the broad substrate specificity and the association of the enzyme with differentiation. In addition, since CS sulfatase is an insoluble microsomal enzyme, contact of the enzyme with CS on the membrane seemed to be limited to give a longer half-life of CS (18). The tissues with high concentrations of CS and low specific activity of cholesterol sulfotransferase, such as gastric and duodenal mucosas, kidney, hair and nail, tended to accumulate CS as a membrane constituent, probably owing to the retarded cleavage of CS by insoluble CS sulfatase. However, the CS sulfatase in hair might be important for regulation of the CS/cholesterol ratio for normal desquamation, as in the stratum corneum (4,19). On the other hand, the tissue distribution of sulfated glycolipids was different from that of CS, being restricted to cerebrum, cerebellum, kidney, testis, and gastrointestinal tract. In the cerebrum and cerebellum, I^3SO_3-GalCer is known to be present in the myelin sheath (20) and to be a molecular marker of oligodendrocytes and Schwann cells (21,22), but its functional significance in other tissues is not clearly understood. However, our finding that the sulfated lipids induced in the human endometrium during the luteal phase and in rabbit endometrium during the pseudopregnancy were I^3SO_3-GalCer and CS, respectively, indicated the correlation of their morphologies, the human endometrium with a well-developed glandular structure containing I^3SO_3-GalCer (23) and rabbit endometrium with a dendritic epithelial structure containing CS (1). This finding coincided with the concentration gradients of CS and I^3SO_3-GalCer in the digestive organs of rabbit, as shown in Tables 1 and 2. The increase in the concentration of I^3SO_3-GalCer from the esophageal to the

jejunal mucosas was compensated for by the decreasing concentration of CS and was proportional to the relative glandular structure contents of the mucosa of the digestive tract. In fact, immunohistochemical staining of the mucosa with the monoclonal anti-I^3SO_3-GalCer antibody revealed the localization of I^3SO_3-GalCer in the apical and basal layers of glands in the gastric, duodenal and jejunal mucosas (Cui Y. and Iwamori, M., unpublished observation). Thus, the tissue-characteristic distribution of sulfolipids indicates the involvement of sulfolipids in the common functions of mammalian tissues.

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Sesamol as an Inhibitor of Growth and Lipid Metabolism in *Mucor circinelloides via* Its Action on Malic Enzyme

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ABSTRACT: Sesamol, a nonoil component of sesame seed oil, inhibited growth, fatty acid synthesis, and desaturation by *Mucor circinelloides in vivo*. Although sesamol also inhibited the growth of other fungi and yeasts, its effect on the lipid metabolism of *M. circinelloides* was exceptional. An enzymological study demonstrated that sesamol affected lipid synthesis primarily by the inhibition of malic enzyme activity, thereby limiting the NADPH supply for fatty acid synthesis and desaturation. Sesamol itself had no inhibitory effect on malic enzyme activity *in vitro*. A metabolite of sesamol is therefore probably responsible for the *in vivo* effects of sesamol on lipid metabolism.
Lipids 32, 605–610 (1997).

Shimizu *et al.* (1), when examining the possible transformation of selected plant oils to more highly unsaturated fatty acids in *Mortierella alpina*, found that sesame seed oil diminished the normally high production of arachidonic acid (20:4) and caused a concomitant appearance of dihomo-γ-linolenic acid (20:3). This observation was explained by there being a nonoil component of the sesame seed oil that was acting as a specific inhibitor of the Δ5 desaturase which would normally have converted 20:3 to 20:4. The active component was subsequently shown to be sesamin (2). Sesamin had little or no effect on other desaturases (Δ6, Δ9, Δ12 desaturases were also studied) in *Mort. alpina*, but was equally effective against Δ5 desaturase activity in rat microsomes. Other nonoil components of sesame seed oil (episesamin, sesaminol, and sesamolin) were also inhibitors of Δ5 desaturases, but sesamol (another nonoil component of sesame seed oil) was not (2). These compounds have in common a methylene- bridged 3,4-dihydroxyphenol moiety and, with the exception of sesamol (which is 3,4-methylenedioxyphenol), are dimeric compounds.

In a study of the effect of plant oils, including sesame seed oil, on other fungi, we found that formation of arachidonic acid in *Entomophthora exitalis* was slightly inhibited by the nonoil component of sesame seed oil (3). It was considered, however, that the active component may have been sesamol and not sesamin (4). A further examination of sesamol has now shown that this compound is particularly inhibitory toward lipid biosynthesis and fatty acid desaturation in *Mucor circinelloides* though not in *E. exitalis*. *Mucor circinelloides* does not produce fatty acids longer than C_{18} and thus does not possess a Δ5 desaturase. It does, however, produce γ-linolenic acid (18:3 n-6) and is therefore of some commercial interest (5).

In this paper, we detail the effects of sesamol on the growth, lipid formation, and fatty acid desaturation in *M. circinelloides* and show that either sesamol, or more likely a derivative of it, causes an inhibition of malic enzyme (E.C. 1.1.1.40) activity. Malic enzyme has previously been shown to be associated with the provision of NADPH for both lipid biosynthesis (6,7) and fatty acid desaturation (6). Therefore, if it can be confirmed that sesamol exerts its effects by the inhibition of malic enzyme, sesamol may prove to be a useful agent by which to control lipid biosynthesis and accumulation.

MATERIALS AND METHODS

Chemicals. All fine chemicals were purchased from Sigma Chemical Corp. (St. Louis, MO) with the exception of sesamol which was purchased from Aldrich Co. Ltd. (Milwaukee, WI). Sodium salts were used throughout, unless otherwise specified.

Cultivation of microorganisms. Mucor circinelloides (CBS 108.16), *E. exitalis* (NRRL 92298), *Aspergillus niger* (ATCC 64108), *Aspergillus flavipes*, *Cryptococcus curvatus* (= *Apiotrichum curvatum* ATCC 20599), *Candida utilis*, *Saccharomyces cerevisiae* (NCYC 738), *Lipomyces starkeyi* (NCYC 1809), and *Rhodosporidium toruloides* (ATCC 226217) were cultivated in 1-L vortex-aerated bottles containing 800 mL of the high (40:1) C:N ratio medium described previously (6). The cultures were incubated at 30°C for 72 h. Biomass was harvested by filtration (filamentous fungi) or centrifugation (yeasts) at 10,000 × *g* for 10 min at 4°C and washed with distilled water.

Preparation of cell extracts. Biomass was suspended in 50 mM phosphate buffer (pH 7.0) containing: 250 mM sucrose, 2 mM benzamidine hydrochloride, and 2 mM dithiothreitol. Disruption of the biomass was achieved by passage, twice,

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through a French press at 35 MPa, and the homogenate was centrifuged at 48,000 × *g* for 20 min at 4°C. The lipid layer was removed by filtration (Whatman No. 1; Maidstone, England) and the protein concentration of the extract determined (8) with bovine serum albumin as a standard.

Estimation of enzyme activities. With the exception of acetyl-CoA carboxylase EC 6.4.1.2, which was assayed using the high-performance liquid chromatography (HPLC) method of De Spiegeleer *et al.* (9), enzyme activities were determined using continuous spectrophotometric assays following the oxidation or reduction of NAD(P)(H) at 340 nm. All assays were carried out at 30°C.

With the exceptions of pyruvate dehydrogenase E.C. 3.1.3.43, pyruvate decarboxylase E.C. 4.1.1.1, and pyruvate carboxylase E.C. 6.4.1.1, the enzymes studied were assayed as described in the literature, with only minor modifications: ATP:citrate lyase E.C. 4.1.3.8 (10), at pH 8.6 with 10 mM sodium azide; malic enzyme E.C. 1.1.1.40 (11); malate dehydrogenase E.C. 1.1.1.37 (12); glucose-6-phosphate dehydrogenase E.C. 1.1.1.49 (13); 6-phosphogluconate dehydrogenase E.C. 1.1.1.44 (14); $NADP^+$/isocitrate dehydrogenase E.C. 1.1.1.42 (15); fatty acid synthetase complex (16).

Pyruvate dehydrogenase was determined using an assay volume containing 300 mM Tris/HCl (pH 8.5), 4 mM L-cysteine, 10 mM pyruvate, 0.12 mM CoA, 1.2 mM NAD^+, and 10 mM azide. Pyruvate decarboxylase was measured using an assay volume containing 50 mM KH_2PO_4/KOH (pH 6.5), 8 mM $MgCl_2$, 1.6 mM cocarboxylase, 2 mM L-cysteine, 0.15 mM NADH, 20 units yeast alcohol dehydrogenase, 5 mM pyruvate, and 10 mM sodium azide. Pyruvate carboxylase activity was determined using an assay volume containing 100 mM Tris/H_2SO_4 (pH 7.8), 3.5 mM ATP, 20 mM $KHCO_3$, 6.7 mM $MgSO_4$, 5 units malic dehydrogenase (from *Thermus flavua*), 84 μM acetyl-CoA, 10 mM pyruvate, 0.15 mM NADH, and 10 mM sodium azide.

Lipid analysis. Freeze-dried biomass was extracted using the method of Folch *et al.* (17) and the cell lipid content determined gravimetrically. Fatty acids were methylated using trimethylsulfonium hydroxide (18) and analyzed by gas chromatography using a packed column, 200 × 0.4 cm, of 10% (w/w) diethylene glycol succinate and 1% (w/w) H_3PO_4 on Chromosorb W-AW (80:100 mesh) (Alltech Associates Inc., Deerfield, IL). The column was run at 200°C with injector and detector at 230°C and N_2 as the carrier gas. Fatty acids were identified by comparison with authentic standards.

Determination of cell dry weights. Ten mL of culture was filtered onto preweighed glass fiber filters, Whatman GF/A (for filamentous fungi), or cellulose acetate filters, 0.45 μm pore size (for yeasts). Filters were washed with distilled water and dried at 100°C to constant weight, and reweighed.

Determination of alcohols in culture filtrates. The culture filtrate was extracted with diethyl ether and the solvent evaporated under reduced pressure to approximately 0.2 mL. The samples were analyzed by gas chromatography using a 5% (w/w) Carbowax 1500 column (on Graphpac 80:100) (Alltech) with N_2 as the carrier gas. The column was at 150°C with the injector and the detector at 175°C. Alcohols were identified by comparison with authentic standards and quantified by reference to a standard curve.

Inclusion of trihydroxybenzenes in enzyme assays. When trihydroxybenzenes were included in enzyme assays, 1 mg superoxide dismutase/mL (from bovine erythrocytes, 2500–7000 units/mg) was included in the assays, and the assays were carried out under N_2 so as to avoid the auto-oxidation of the trihydroxybenzenes.

Thin-layer chromatography. Thin-layer chromatography analysis was carried out on silica G-gel plates (0.25-mm thick, 20 × 20 cm; Merck, Darmstadt, Germany). The mobile phase contained chloroform/methanol/water/acetic acid (65:43:3:1, by vol) and spots were visualized by ultraviolet illumination.

Reproducibility of experimental data. All experiments were carried out on three separate occasions (unless otherwise stated), and the data obtained demonstrated reproducible trends in all cases. Owing, however, to the variation in the absolute values obtained in different experiments, representative data from single experiments are presented.

RESULTS AND DISCUSSION

Analysis of commercial sesamol. Thin-layer chromatographic analysis of commercial sesamol showed that it contained approximately 7.5% (w/w) impurities. Because these impurities, when isolated and tested separately, had no effect on the growth or lipid metabolism of *M. circinelloides*, the data presented here were obtained using commercial sesamol without further purification.

Effect of sesamol on the growth and production of lipid by M. circinelloides. Sesamol inhibited both the growth of and lipid production by *M. circinelloides* (see Table 1). Growth inhibition was evident at 1.5 mM sesamol, and at 10 mM was complete. The production of lipid by *M. circinelloides* was more sensitive to the presence of sesamol than cell growth. While the inclusion of 1.5 mM sesamol in culture medium caused less than a 10% decrease in fungal biomass, the lipid content of the mycelia was decreased by 75%. Increased concentrations further inhibited lipid production so that mycelia grown in the presence of greater than 4 mM sesamol contained only 1 to 2% (w/w) cell lipid. Since vegetatively growing *M. circinelloides* (in the absence of sesamol) contained approximately 5% (w/w) cell lipid (data not presented), it is probable that 1 to 2% (w/w) cell lipid represents the minimum lipid content that allowed cell survival.

Sesamol not only affected cell lipid accumulation quantitatively in *M. circinelloides*, but also the fatty acyl composition of the cell lipid produced (see Table 1). The 18:3 n-6 content of the cell lipid was most affected, being decreased by 50% in the presence of 1.5 mM sesamol with a concomitant increase in the content of linoleic acid (18:2). This decrease in the 18:3 n-6 content of the cell lipid was indicative of an inhibition of the Δ6 desaturase activity. As the concentration of sesamol was increased, the contents of both 18:3 n-6 and

TABLE 1
Effect of Sesamol on the Growth, Lipid Production, and Fatty Acyl Composition of Cell Lipid in *Mucor circinelloides*

	Sesamol added to medium (mM)							
	0.0	1.5	2.9	4.3	5.6	7.2	8.7	10.1
Cell dry wt (g/L)	10.2	9.4	8.5	7.7	5.8	4.8	1.6	0.2
Cell lipid content (% w/w dry weight)	24	5	3	2	2	1	1	2
Fatty acyl group	Fatty acyl composition of cell lipid (relative % w/w)							
16:0	18	16	13	12	11	13	11	12
18:0	8	9	26	11	17	10	14	14
18:1	40	40	46	52	53	55	56	63
18:2	12	19	5	10	8	8	6	2
18:3n-6	16	8	2	3	2	5	4	3
Others[a]	6	8	7	12	9	9	9	6
UI	1.14	1.07	1.10	0.88	0.78	0.99	0.84	0.80

[a]Others = 14:0, 14:1, 14:2, 16:1, and 16:2, all were present at less than 0.5% (w/w) total cell lipid; UI = unsaturation index = [% monoene + 2(% diene) + 3(% triene)]/100.

18:2 decreased, and there was a gradual increase in the content of oleic acid (18:1) (to over 150% its initial value) and stearic acid (18:0) (to approximately twice its initial value). The increase in the content of saturated and monounsaturated fatty acids in the cell lipids, at the expense of the di- and triunsaturated fatty acids, indicated that sesamol was inhibiting both the Δ6 and Δ12 desaturases, even though to different extents, and suggested, because of the increase in 18:0, that the Δ9 desaturase may also be affected. That the inhibition of all three desaturases was occurring distinguishes the action of sesamol from that of the other nonoil components of sesame seed oil that have been shown to specifically inhibit the Δ5 fatty acid desaturase in other systems (2).

Supplementation of the culture medium with sesamol affected the intermediary metabolism of *M. circinelloides*, promoting the accumulation of alcohols by the fungus. The most abundant alcohols produced were ethanol (22 mM) and butan-2-ol (4 mM), giving a combined yield of approximately 1.5 g/L. No alcohol was detected in the culture filtrates of *M. circinelloides* grown in the absence of sesamol. A similar diversion of carbon from lipid production to ethanol production has been reported to occur in *Fusarium moniliforme* when exposed to high concentrations of Zn^{2+} (19).

Effect of sesamol on the growth and lipid production by a range of fungi and yeasts. The addition of sesamol to the culture medium inhibited the growth of all the microorganisms examined (see Table 2), with 7.2 mM sesamol causing total inhibition in all cases. In this regard the effect of sesamol on these organisms appeared even more pronounced than that on *M. circinelloides*. However the amount of cell lipid accumulated by these microorganisms was not affected by sesamol, even at concentrations that severely inhibited growth (data not presented), indicating that sesamol affected neither lipid biosynthesis nor fatty acid desaturation in these organisms. Thus the action of sesamol on the lipid metabolism of *M. circinelloides* appeared to be exceptional.

Effect of sesamol on the activity of key enzymes in vivo. To establish the mode of action of sesamol at the enzymic level, the activities of a number of enzymes were determined in *M. circinelloides* grown in the presence and absence of 5.7 mM sesamol (see Table 3). Of the enzymes detected, significant changes were observed only in the activities of pyruvate carboxylase (decreased by 85%), pyruvate decarboxylase (increased by 300%), $NADP^+$/isocitrate dehydrogenase (decreased by 57%), and malic enzyme (decreased by 98%). Pyruvate dehydrogenase and acetyl-CoA carboxylase activities were assayed but not detected in any extract of *M. circinelloides*. The failure to detect these two enzymes was presumably due to their lability and deactivation during the production of cell extracts rather than the absence of these enzymes in *M. circinelloides*.

The changes in the activities of pyruvate carboxylase, which was 85% inhibited, and pyruvate decarboxylase, which was stimulated 3-fold, can explain the accumulation of ethanol when *M. circinelloides* was grown in the presence of sesamol. Pyruvate carboxylase is one of the two enzymes (the other being pyruvate dehydrogenase) involved in channeling pyruvate into the citric acid cycle. Pyruvate decarboxylase, on the other hand, catalyzes the conversion of pyruvate to acetaldehyde, which is subsequently converted to ethanol. Thus the decrease in pyruvate carboxylase activity (which would limit citrate production) and the increase in pyruvate decarboxylase activity (leading to acetaldehyde accumulation) would lead to a diversion of carbon, at the level of pyruvate, from the citric acid cycle to acetaldehyde and subsequently into ethanol production.

The decreases in $NADP^+$/isocitrate dehydrogenase and malic enzyme activity both will affect the NADPH-generating capacity of the fungus and therefore may be responsible for the changes in lipid metabolism observed when *M. circinelloides* is exposed to sesamol. It is likely that the decrease in malic enzyme activity was the most important fac-

TABLE 2
Effect of Sesamol, in the Culture Medium, on Growth and Lipid Production in Various Yeasts and Fungi[a]

Microorganism	Sesamol in culture medium (mM)	Cell dry weight (g/L)	Cell lipid (% w/w cell dry weight)
Entomophthora exitalis	0	6.624	
	0.2	3.6	23
	0.9	1.4	21
	2.0	0.0	—
Cryptococcus curvatus	0	9.1	36
	0.35	7.2	29
	0.72	6.0	44
	3.5	0	—
	7.2	0	—
Candida utilis	0	4.0	2
	0.35	3.8	4
	0.72	3.8	2
	3.5	3.0	3
	7.2	0.3	4
Rhodosporidium toruloides	0	8.1	29
	0.35	4.6	33
	0.72	3.2	27
	3.5	1.2	36
	7.2	0	—
Lipomyces starkeyi	0	2.6	20
	0.35	3.0	16
	0.72	2.7	14
	3.5	3.2	18
	7.2	0.0	—
Saccharomyces cerevisiae	0	2.2	3
	0.35	2.4	2
	0.72	2.5	2
	3.5	1.6	3
	7.2	0	—
Aspergillus flavipes	0	3.3	5
	0.35	3.0	7
	0.72	2.8	7
	3.5	2.4	6
	7.2	0	—
Aspergillus niger	0	4.6	2
	0.35	4.0	5
	0.72	3.6	2
	3.5	3.0	1
	7.2	0	—

[a]The microorganisms were grown for 72 h in vortex-aerated bottles prior to determination of the cell dry weights and cell lipid contents.

tor affecting the lipid metabolism of the fungus as the change in this activity was the most dramatic. Also malic enzyme has been shown to be an important source of NADPH for the production of storage lipid in oleaginous fungi (6,7) and for fatty acid desaturation in *M. circinelloides* (6). Hence the almost complete absence of malic enzyme activity in *M. circinelloides* when the fungus was grown in the presence of sesamol would explain the inhibition of both lipid accumulation and fatty acid desaturation.

Effect of sesamol on enzyme activities in vitro. When 5 mM sesamol was added directly to the enzyme assays, it did not affect the activity of any of the enzymes given in Table 3. Even at 50 mM, sesamol did not inhibit malic enzyme activity. This lack of effect *in vitro* suggested that the action of sesamol on the activity of malic enzyme, $NADP^+$/isocitrate dehydrogenase, pyruvate carboxylase, and pyruvate decarboxylase in growing cells (see Table 3) may not be due to the presence of sesamol itself but to a product of sesamol formed by its metabolism by *M. circinelloides*.

An alternative suggestion, that the decreases in the activities of malic enzyme, $NADP^+$/isocitrate dehydrogenase and pyruvate carboxylase could be due to repression of the synthesis of these enzymes by sesamol or a metabolite of sesamol, was precluded. *Mucor circinelloides* was grown for 16 h in the absence of sesamol. Biomass was harvested, washed, and transferred to a medium containing either glucose (supplemented with 5 mM sesamol) or acetate (10 g/L, containing no sesamol) as the carbon source. Acetate represses malic en-

TABLE 3
Effect of Inclusion of 5.8 mM Sesamol, in the Culture Medium, on the Activities of Selected Enzyme Activities in *Mucor circinelloides*[a]

Enzyme	Enzyme-specific activities [nmol·(min·mg protein)$^{-1}$]	
	Control culture (no sesamol)	Culture supplemented with 5.8 mM sesamol
ATP/citrate lyase	27	31
Pyruvate decarboxylase	58	164
Pyruvate carboxylase	246	32
Malic enzyme	149	3
Glucose-6-phosphate dehydrogenase	159	197
6-Phosphogluconate dehydrogenase	140	147
$NADP^+$/Isocitrate dehydrogenase	301	129
Malate dehydrogenase	7400	6300
Fatty acid synthetase	14	11

[a]The experiment was carried out in quadruplicate, and representative data are presented.

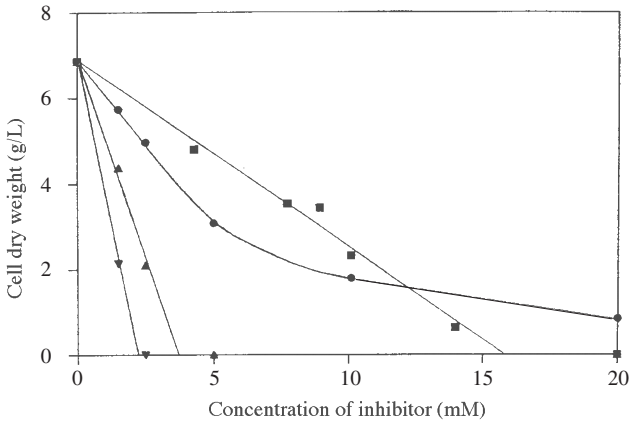

FIG. 1. Effect of sesamol and sesamol analogs on the growth of *Mucor circinelloides*. Methylenedioxybenzene-containing compounds were included in the culture medium up to 20 mM: sesamol (■), piperonyl butoxide (●), safrole (▲), and isosafrole (▼).

zyme activity in *Neurospora crassa* (20) and in *M. circinelloides* (data not presented). Transfer of biomass to a medium containing sesamol (with glucose as the carbon source) caused a 45% decrease in the activity of malic enzyme after 60 min, whereas there was no change in malic enzyme activity in biomass transferred to a medium containing acetate after 60 min. Repression of the malic enzyme activity in the acetate medium was not observed until after 3.5 h.

When the cell extracts produced from the cells exposed to sesamol for 1 h were dialyzed, malic enzyme activity was not restored, suggesting that the inhibition of malic enzyme activity involved an irreversible effect, with the active component either binding to the enzyme or causing posttranscriptional changes within its structure.

Effect of compounds containing a methylenedioxybenzene group on the growth of, and lipid production by, M. circinelloides. It was thought possible that the active breakdown product of sesamol, responsible for the inhibition of cell growth and effects on lipid metabolism, may contain a methylenedioxybenzene structure. Such compounds have long been recognized as being inhibitors of microsomal oxidases in mammalian and insect systems (21–23).

The inclusion of safrole, isosafrole, and piperonyl butoxide (all compounds containing a methylenedioxybenzene group) in the culture medium also inhibited growth. Indeed all three compounds were equally, or more, inhibitory to growth of *M. circinelloides* than sesamol (see Fig. 1). However, none of these compounds adversely affected lipid biosynthesis, fatty acid desaturation or, most significantly, malic enzyme activity in *M. circinelloides* (data not presented) and were therefore not thought to resemble the active metabolite responsible for the *in vivo* inhibition of malic enzyme activity.

Effect of a range of catechol derivatives on the activity of malic enzyme. Another possibility was that sesamol may undergo the removal of its methylene C atom to yield a catechol-like compound. Catechol-like compounds are formed (*in vivo*) from methylenedioxyphenyl compounds when these compounds are administered to both animals and insects (24). A range of catechol derivatives was therefore tested for their ability to inhibit malic enzyme activity *in vitro* (see Table 4).

Several of the compounds were potent inhibitors of malic enzyme *in vitro*, the most potent (1,2,3-trihydroxybenzoic acid) causing 50% inhibition of the activity (IC_{50} value) at 0.22 mM. The inhibition of malic enzyme activity appeared to depend upon the possession of two hydroxyl functions on adjacent carbons of the benzene ring. 1,3,5-Trihydroxybenzoic acid, a compound that possesses three hydroxyl groups separated from each other on the benzene ring by an unhydroxylated carbon, did not cause inhibition of malic enzyme

TABLE 4
The Apparent IC_{50} Values for Sesamol and a Range of Catechol-Related Compounds with Respect to Malic Enzyme Activity Assayed *in vitro*[a]

Compound	Apparent IC_{50} value (mM)
Sesamol	No inhibition
Catechol	0.5
3-Methyl catechol	3.1
4-Methyl catechol	15.9
3-Isopropyl catechol	0.9
4-Tertiary butyl catechol	5.6
3,4-Dihydroxybenzoic acid	16
1,2,4-Trihydroxybenzoic acid	0.37
1,2,3-Trihydroxybenzoic acid	0.22
1,3,5-Trihydroxybenzoic acid	No inhibition
2-Hydroxybenzyl alcohol	2.8
3-Hydroxybenzyl alcohol	No inhibition
4-Hydroxybenzyl alcohol	No inhibition
Resorcinol	23.8
2,3-Dihydroxybiphenol	No inhibition

[a]IC_{50} value (inhibitory concentration 50%) was the concentration of each compound that resulted in the reduction in the apparent activity of malic enzyme to 50% of its initial value.

activity. The dialysis of extracts after the addition of the catechol-related inhibitors did not reinstate malic enzyme activity, demonstrating that the inhibition caused by these compounds was, like that of sesamol itself (when activated in the cell), irreversible.

Although catechol, 3-methyl catechol, and 2-hydroxybenzyl catechol were inhibitors of malic enzyme *in vitro*, none of these caused a decrease in malic enzyme activity *in vivo* when added to actively growing cells of *M. circinelloides*. This was attributed to these compounds not accumulating within the fungal cells, owing to either a lack of uptake or the rapid metabolism of these compounds within the cells.

It seems likely therefore that sesamol is a compound that, although it is not an inhibitor of malic enzyme itself, is readily assimilated by *M. circinelloides.* Once assimilated it is metabolized to form a catechol-like compound which inhibits malic enzyme activity and causes the inhibition of both fatty acid accumulation and desaturation by limiting the supply of NADPH.

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Modification of Odd-Chain Length Unsaturated Fatty Acids by Hepatocytes of Rainbow Trout (*Oncorhynchus mykiss*) Fed Diets Containing Fish Oil or Olive Oil

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ABSTRACT: Hepatocytes isolated from rainbow trout fed on diets containing either fish oil or olive oil were incubated with individual odd-chain length unsaturated fatty acids (19:1n-9, 19:2n-6, 19:3n-3, 21:2n-6, 21:3n-6, 21:4n-6, 21:3n-3, and 21:5n-3) to examine whether these fatty acids were substrates for modification by desaturation and elongation. All odd-chain length fatty acids were readily assimilated into the lipids of hepatocytes from both dietary groups of fish, but their conversion to longer-chain, more unsaturated derivatives was more pronounced with cells from trout fed olive oil. Thus, the conversion of 19:2n-6 and 21:2n-6 to 21:3n-6 and 21:4n-6, and of 19:3n-3 to 21:4n-3 and 21:5n-3, was most obvious in cells from the olive oil group, as was the conversion of 21:3n-6 and 21:3n-3 to 21:4n-6 and 21:4n-3, respectively. Elongation of 19:1n-9 to 21:1n-9 and 23:1n-9 occurred in cells from both groups. No 23:6n-3 was detectable as a product of 19:3n-3 or 21:3n-3. However, this fatty acid was a major product formed by cells from fish fed olive oil presented with 21:5n-3. Cells from both groups of fish incorporated 21:4n-6 and 21:5n-3 into their lipids largely without modification but chain-shortened around 40, 23, and 19% of the incorporated 21:2n-6, 21:3n-3, and 19:1n-9, respectively. The results demonstrate that odd-chain length unsaturated fatty acids can act as substrates for the desaturation, elongation, and chain-shortening systems of trout hepatocytes.
Lipids 72, 611–619 (1997).

Fish, in common with other animals, are incapable of synthesizing polyunsaturated fatty acids (PUFA) *de novo* but can, depending on species, convert to a greater or lesser extent PUFA arising in the diet to their longer and usually more unsaturated counterparts (1). Despite the natural abundance of 22:6n-3 in the lipids of fish (2), little is known of how it is formed, although evidence gained from recent studies with trout hepatocytes (3) indicates that it is independent of a Δ4 desaturase and proceeds *via* C_{24} intermediates as has been established for rats (4).

The liver plays a critical role in various aspects of lipid metabolism such as the uptake, oxidation, and conversion of fatty acids and the supply of long-chain PUFA to the other tissues (5,6). Thus, isolated hepatocytes have proved to be a good metabolic model for the study of lipogenesis in fish (7).

Most previous studies on the biosynthetic pathways of PUFA in isolated fish cells, e.g. (3,8), have employed radiolabeled fatty acids as substrates but, although much information has been gained, details of the individual conversion steps have not been fully established. In early studies on the biosynthesis of n-6 PUFA in rat, the odd-chain length PUFA 17:2n-5 and 19:2n-7 were used to help establish the sequence of desaturation and elongation steps involved in the addition of two more double bonds (9). By analogy, odd-chain length unsaturated fatty acids (OCLUFA) can also be used to investigate the formation of the highly unsaturated fatty acids containing five or six double bonds in fish liver cells. Apart from the occurrence of very low levels of 21:5 in the lipids of some marine organisms (10–12) and of some n-6 odd-chain components in rat seminiferous tubules (13), odd-chain PUFA are very rare in nature. Thus, the use of OCLUFA as tracers for the enzymes of conversion should not be affected by the dilution of products by existing fatty acids. Although [1-^{14}C]- and, more recently, [3-^{14}C]-radiolabeled fatty acids have been used successfully to gain information on the pathways of PUFA formation in animal cells (3,14–16), the loss of their radiolabel after one or two cycles of β-oxidation makes it difficult to study the final fate of the substrate. The use of odd-chain length fatty acids as substrates does not have this disadvantage, since the actual mass of the substrates and their products in cell lipid can be measured.

The present study was undertaken to examine whether OCLUFA can help elucidate PUFA formation in trout hepatocytes. For this purpose, eight different odd-chain length substrates were synthesized, presented to hepatocytes, and the cell fatty acid composition examined for various products. Since it has been shown recently that the rate of 22:6n-3 synthesis from 18:3n-3 in trout hepatocytes *in vitro* is enhanced by maintaining the fish on a diet devoid of preformed long-

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Abbreviations: BSA, bovine serum albumin; FAME, fatty acid methyl esters; FCS, fetal calf serum; GC, gas chromatography; HBSS, Hank's balanced salt solution; HPTLC, high-performance thin-layer chromatography; MS, mass spectrometry; OCLUFA, odd-chain length unsaturated fatty acids; OO, olive oil; PL, polar lipids; PUFA, polyunsaturated fatty acids; TAG, triacylglycerols; TL, total lipids; TLC, thin-layer chromatography.

chain n-3 PUFA (3), the uptake and modification of the odd-chain length substrates by hepatocytes prepared from trout maintained on diets containing fish oil or olive oil were compared.

EXPERIMENTAL PROCEDURES

Materials. Medium 199 (M199), Hank's balanced salt solution (HBSS), fetal calf serum (FCS), fatty acid-free bovine serum albumin (BSA), collagenase Type IV, antibiotics (penicillin/streptomycin, 5000 units/5 mg mL^{-1}), and the anesthetic 3-aminobenzoic acid ethyl ester were all obtained from Sigma (Poole, Dorset, United Kingdom).

Thin-layer chromatography (TLC) (20 × 20 cm × 0.25 mm) and high-performance thin-layer chromatography (HPTLC) (10 × 10 cm × 0.15 mm) plates precoated with Silicagel 60 (without fluorescent indicator) were purchased from Merck (Darmstadt, Germany). All organic solvents were of high-performance liquid chromatography grade and were purchased from Rathburn Chemicals (Walkerburn, Scotland, United Kingdom).

Experimental animal and diets. Juvenile rainbow trout, *Oncorhynchus mykiss* (70–100 g), were obtained from a commercial fish farm and maintained as two groups in 60-L self-cleaning circular tanks, with constantly flowing water at a temperature of 12°C. One group was fed on a control commercial diet containing fish oil (EWOS, Bathgate, United Kingdom) while the other was maintained on an experimental diet based on olive oil (OO) and deficient in both 20:5n-3 and 22:6n-3 (3). Fish were fed at a daily rate of 2% of their body weight and killed after 3 mon to prepare the hepatocytes.

Preparation of hepatocytes. Trout hepatocytes were prepared by modification of the methods of Moon *et al.* (7) and Klaunig *et al.* (17). Anesthetized fish were killed by a sharp blow to the head, and their livers rapidly removed. The hepatic portal vein or another suitable vessel was then cannulated to clear the liver of blood by perfusion with well-oxygenated (95% O_2/5% CO_2) HBSS, which contained 0.6 mM EDTA, 10 mM HEPES, and 25 mM $NaHCO_3$. The liver was then subjected to a second perfusion with HBSS containing 10 mM HEPES, 4 mM $CaCl_2$, 25 mM $NaHCO_3$ and 1 mg/mL of collagenase to yield a well-digested liver after 30–45 min. Poorly perfused areas were removed and the rest passed through a nylon screen of plankton netting (250 μm). Cells were collected by centrifugation at 100 × *g* for 5 min and washed twice with HBSS containing 10 mg/mL of BSA. The isolated hepatocytes were gently resuspended into M199 containing 2% of FCS and 1% of the antibiotic mixture. A sample was taken to establish the number and viability of cells by using trypan blue exclusion. Protein content of the hepatocytes was determined according to Lowry *et al.* (18).

Synthesis of odd-chain length unsaturated fatty acids. Eight odd-chain length fatty acids, 19:1n-9 [10-19:1], 19:2n-6 [10,13-19:2], 19:3n-3 [10,13,16-19:3], 21:2n-6 [12,15-21:2], 21:3n-6 [9,12,15-21:3], 21:3n-3 [12,15,18-21:3], 21:4n-6 [6,9,12,15-21:4], and 21:5n-3 [6,9,12,15,18-21:5], were synthesized from their respective C_{18} and C_{20} fatty acids as follows. The fatty acid (0.1 g) dissolved in anhydrous diethyl ether (10 mL) was added dropwise over 10 min to a magnetically stirred solution of an excess of lithium aluminum hydride in anhydrous ether (0.3 g/10 mL) under an atmosphere of argon. The solution was stirred for 4 h, and then a solution of saturated ammonium chloride (5 mL) added dropwise to destroy excess lithium aluminum hydride. The ether phase was separated, the residual aqueous phase washed with diethyl ether (3 × 10 mL), and the combined ether extracts dried over anhydrous magnesium sulfate, filtered, and evaporated to yield the crude alcohol.

The crude alcohol was dissolved in dry dichloromethane, and methane sulfonyl chloride (0.1 g) and 1 mL of pyridine were added to the solution which was then stirred overnight at room temperature under an atmosphere of argon. The dichloromethane was removed by evaporation under reduced pressure and the residue dissolved in diethyl ether (50 mL). The ether solution was extracted with 2 M HCl (4 × 25 mL), and the combined HCl extracts were back-extracted with ether (3 × 25 mL). The combined ether extracts were dried over anhydrous magnesium sulfate, filtered, and evaporated. The resultant crude mesylate was purified by flash column chromatography on silica, using a petroleum ether/ethyl acetate gradient (0–10%) to yield a homogeneous product as assessed by TLC using petroleum ether/ethyl acetate (9:1, vol/vol) as developing solvent.

To the purified mesylate dissolved in 10 mL of dry dimethylformamide, potassium cyanide (0.15 g, 97%) was added and the reaction mixture stirred at 80°C in the dark for 24 h. The reaction mixture was poured onto water (200 mL) and extracted with diethyl ether (6 × 50 mL). The combined ether extracts were backwashed with saturated sodium chloride solution, dried over $MgSO_4$, filtered, and evaporated to dryness on a rotary evaporator. Following purification by flash column chromatography as described above, the crude product was chromatographically homogeneous and displayed a characteristic nitrile stretch in the infrared spectrum at *ca.* 2245 cm^{-1}.

Finally, the purified nitrile was stirred overnight with a solution of 25% anhydrous hydrochloric acid in anhydrous methanol (10 mL) under an atmosphere of argon. The methanol was removed by rotary evaporation and the residue partitioned between water (10 mL) and diethyl ether. The ether layer was separated, and the aqueous phase washed with ether (3 × 25 mL). The combined ether extracts were dried, filtered, and evaporated to yield the crude fatty acid methyl esters (FAME) which were purified by flash column chromatography as described above. The product was chromatographically homogeneous and displayed characteristic ester carbonyl (1730 cm^{-1}) and double-bond (1650 cm^{-1}) stretches in the infrared spectrum.

Purity of the FAME product was assessed by gas chromatography (GC) as described below and was in excess of 97% for all the odd-chain length fatty acids. The identity of

individual OCLUFA was confirmed by GC–mass spectrometry (GC–MS) of their methyl esters using the system described below. After hydrolysis of the FAME (19), HPTLC analysis showed the products to be entirely in their free fatty acid form. For presentation to hepatocytes, the fatty acids were converted to their potassium salts by dissolving in a 10-fold molar excess of 0.1 M KOH solution followed by the addition to the solution of sufficient BSA to give a molar ratio of fatty acid to BSA of 6:1.

Hepatocyte incubation. Isolated hepatocytes were incubated as monolayers in sterile plastic Petri dishes at a concentration of 5.5×10^6 cells mL^{-1}, in 5 mL of M199 supplemented with FCS and antibiotics. The cells were incubated for 18 h at 10°C with 30 μM of one of the OCLUFA, which were added to the medium as their potassium salts bound to BSA. The incubation conditions were established in preliminary experiments which proved that a substrate concentration of 30 μM with an incubation time of 18 h yielded high fatty acid recoveries without adversely affecting the lipid class composition and cell viability. A control group of cells was also maintained under the same conditions without the addition of any fatty acid to their incubation media.

After 18 h of incubation, hepatocytes were aspirated into test tubes and subjected to the trypan blue viability test. Viability rate was not significantly affected ($P < 0.05$) by the addition of any of the OCLUFA in either dietary group and routinely exceeded 95%.

Lipid extraction. Hepatocytes were washed twice with M199 in test tubes and their lipids extracted with chloroform/methanol (2:1, vol/vol) as described by Christie (20). The organic solvent was evaporated under a stream of nitrogen and the lipid content determined gravimetrically.

Lipid class analysis. Lipid classes were separated by one-dimensional, double development HPTLC using methyl acetate/isopropanol/chloroform/methanol/0.25% (wt/vol) KCl (25:25:25:10:9, by vol) as the polar solvent system, and hexane/diethyl ether/glacial acetic acid (80:20:2, by vol) as the neutral solvent system. Lipid classes were quantified by charring with copper acetate reagent followed by calibrated scanning densitometry using a Shimadzu CS-9000 dual wavelength flying spot scanner and DR-2 recorder (Tokyo, Japan) (21).

Fatty acid analysis. The polar lipid (PL) fraction and triacylglycerols (TAG) were separated by TLC using hexane/diethyl ether/glacial acetic acid (80:20:2, by vol) as the developing solvent. Chromatograms were sprayed with 2′,7′-dichlorofluorescein in 98% (vol/vol) methanol containing 0.01% (wt/vol) butylated hydroxytoluene and the separated lipids visualized under ultraviolet light.

Bands of adsorbent containing the PL fraction and TAG were scraped off the plates into test tubes and subjected to acid-catalyzed transmethylation to yield FAME (19). A portion of the total lipid (TL) was also subjected to the transmethylation procedure. Prior to transmethylation, 19:0 fatty acid was added as an internal standard to the TL, TAG, and PL fractions.

The analysis of FAME was performed using a Packard 436 gas chromatograph equipped with a capillary column of fused silica (25 m × 0.32 mm i.d.) coated with FFAP CB (Chrompack, Middleburg, The Netherlands). Hydrogen was used as carrier gas, and during each analysis the oven temperature was programmed to rise from 50 to 150°C at a rate of 39°C min^{-1} and then 2.5°C min^{-1} to a final temperature of 230°C. Individual FAME were identified by reference to authentic standards and to a composite standard prepared by mixing a well-characterized fish oil and the eight OCLUFA used in incubations with hepatocytes. To confirm the identity of the fatty acids derived from OCLUFA substrates, FAME prepared from hepatocyte lipids were subjected to analysis by GC–MS using a Carlo Erba 8000 GC coupled to a MD 800 MS (Fisons Instruments, Crawley, United Kingdom). The gas chromatograph was equipped with a fused-silica capillary column (15 m × 0.25 mm i.d.) coated with DB 5MS (J&W Scientific, Folsom, CA) and helium used as carrier gas. Samples were applied using on-column injection with the oven temperature programmed to rise from 50 to 225°C.

Statistical analysis. Data are presented as means of triplicates ± SD. For analysis by GC–MS of products derived from OCLUFA, triplicates of FAME samples were pooled and for this reason data corresponding to these products are expressed as a single value. Pairs of means were compared by a two-tailed Student's *t*-test. Comparisons between a mean and an undetected fatty acid were performed using a one-tailed *t*-test considering the absent fatty acid as zero.

Homogeneity of the OCLUFA recoveries was checked by using the Bartlett's variance test and when necessary data were normalized by arcsin transformation. Differences between the eight means were analyzed by one-way analysis of variance followed, where appropriate, by Tukey's multiple comparison test (22).

In all statistical tests used, *P* values of less than 0.05 were considered statistically different.

RESULTS

Lipid class composition of trout hepatocytes. Hepatocytes isolated from trout fed OO diet had a significantly higher lipid content than those prepared from fish maintained on the control diet (Table 1). Although differences were observed between the two dietary groups in hepatocyte lipid class composition, these were generally minor and the overall proportions of total polar and total neutral lipids were not significantly different between the two groups (Table 1). TAG and choline glycerophospholipids were always the major neutral and PL, respectively. The addition of OCLUFA to the incubation medium did not affect the lipid class composition of the hepatocytes (data not shown).

Fatty acid composition of dietary lipids and trout hepatocytes. The level of n-3 fatty acids present in the OO diet was very low in comparison with that of the control diet (0.9 vs. 19.4% of total fatty acids). More than 70% of the fatty acid in the OO diet was oleic acid (18:1n-9) with the rest being mainly palmitic (16:0) and linoleic (18:2n-6) acids (Table 2).

TABLE 1
Total Lipid Content and Lipid Class Composition of Hepatocytes[a] Isolated from Trout Fed the Control and the Olive Oil Diets

	Control	Olive oil
Total lipid ($\mu g/10^6$ cells)	50.7 ± 5.8	78.5 ± 11.1*
Lipid class (% total lipid)		
LPC	0.5 ± 0.1	0.5 ± 0.1
SM	3.9 ± 0.4	2.8 ± 0.4*
CGP	30.7 ± 2.3	29.1 ± 1.8
PS	4.2 ± 0.3	3.4 ± 0.5*
PI	6.3 ± 0.4	7.5 ± 0.5*
EGP/CL/PG	17.9 ± 2.3	21.1 ± 2.7*
Total polar lipids	63.6 ± 2.4	64.4 ± 2.7
Chol	10.3 ± 0.7	12.1 ± 1.6*
FFA	1.1 ± 0.4	0.8 ± 0.4*
TAG	20.8 ± 2.8	20.5 ± 3.3
SE	2.4 ± 0.6	1.1 ± 0.6*
Unidentified	1.8 ± 0.4	1.0 ± 0.4*
Total neutral lipids	36.4 ± 2.4	35.5 ± 2.7

[a]After 18h of incubation. Data are means ± SD ($n = 3$ fish for total lipid content and $n = 12$ fish for lipid class composition). Means within each row that were significantly different ($P < 0.05$) are shown by an asterisk (*).
Abbreviations: LPC, lysophosphatidylcholine; SM, sphingomyelin; CGP, choline glycerophospholipids; PS, phosphatidylserine; PI, phosphatidylinositol; EGP, ethanolamine glycerophospholipids; CL, cardiolipin; PG, phosphatidylglycerol; Chol, cholesterol; FFA, free fatty acids; TAG, triacylglycerols; SE, steryl esters.

TABLE 2
Fatty Acid Composition (wt%) of Diets Fed to Trout

	Diets	
Fatty acids	Control	Olive oil
14:0	6.7	trace
15:0	0.5	trace
16:0	13.2	11.8
16:1n-9 + n-7	4.8	1.4
16:2	0.7	—
16:3	0.5	—
18:0	1.8	2.9
18:1n-9 + n-7[a]	14.3	72.1
18:2n-6	5.0	9.6
18:3n-3	1.6	0.6
18:4n-3	2.7	—
20:1n-11 + n-9	11.8	0.4
20:4n-6	0.4	trace
20:4n-3	0.6	trace
20:5n-3	5.9	trace
22:1n-11 + n-9	16.0	0.4
22:5n-3	0.8	trace
22:6n-3	7.8	trace
Total saturates	22.2	14.7
Total monoenes	46.9	74.3
Total PUFA[b]	26.7	11.0
Total n-6	6.1	9.8
Total n-3	19.4	0.9
n-6/n-3	0.3	10.9
Unidentified	4.2	—

[a]Mainly 18:1n-9 isomer.
[b]Also includes 20:2n-6, 22:4n-6, and 22:5n-6. PUFA, polyunsaturated fatty acids; —, not detected; trace, fatty acid values < 0.4%.

The fatty acid compositions of hepatocytes from trout fed either the control or the n-3-deficient OO diet are shown in Table 3. The proportions of n-3 PUFA present in the TL decreased from 48.7% in the control cells to 21.7% in the hepatocytes from trout fed the OO diet. Conversely, these latter hepatocytes contained approximately double the levels of monounsaturated fatty acids and n-6 fatty acids in their TL than those from the control group.

The depletion of n-3 PUFA, particularly 22:6n-3 and 20:5n-3, in the hepatocytes of the OO dietary group was less pronounced in the PL fraction than in the TAG. The increments of n-9 fatty acids observed in both TAG and PL of these hepatocytes were caused mainly by the accumulation of 18:1n-9 together with the appearance of 20:2n-9 and especially 20:3n-9. At the same time, the total level of n-6 fatty acids increased, particularly in the PL, owing to increases in the proportions of 18:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, and 22:5n-6.

Incorporation of OCLUFA in trout hepatocytes lipids. The amounts of unmodified substrates recovered in the lipids of hepatocytes prepared from fish fed the control and OO diets are shown in Table 4. With the exception of 21:3n-6 and 21:3n-3, the amount of each unmodified OCLUFA recovered in the TL and TAG of cells was not significantly influenced by the diet fed to the fish. Hepatocytes from trout maintained on the OO diet assimilated less 21:3n-6 into TL and less 21:3n-3 into TAG than those from fish fed the control diet. An increased incorporation of OCLUFA substrates under n-3-deficient conditions was more evident in PL although this increment was only statistically significant for 19:2n-6, 21:4n-6, 21:3n-3, and 21:5n-3.

Notable differences existed between the eight substrates in terms of absolute amounts recovered in hepatocyte lipids (Table 4). With cells from both dietary groups, the levels of 19:3n-3 recovered in TL were significantly higher than those of 21:3n-3 or 21:2n-6. Differences between the substrates were less pronounced in the TAG, although the amounts of 19:3n-3 and 21:5n-3 recovered in this lipid class were always statistically higher than those of 21:3n-3. The recovery patterns of the OCLUFA substrates in hepatocyte PL differed between the two dietary groups. In hepatocytes from control fish significantly higher amounts of both 19:1n-9 and 19:3n-3 than 21:2n-6 and 21:3n-3 were present, whereas in the n-3-deficient cells recoveries of 19:3n-3, 21:4n-6, and 21:5n-3 in PL were higher than those of 21:2n-6, 21:3n-6, and 21:3n-3.

OCLUFA conversion products. The lipids of hepatocytes incubated with OCLUFA substrates contained measurable amounts of other OCLUFA derived from these substrates by chain-shortening, elongation, and desaturation (Tables 5–7). Thus, C_{17}, C_{21}, and C_{23} monounsaturated fatty acids were detected in cells incubated with 19:1n-9, and it was notable that 17:1 was always more abundant than 21:1 and 23:1, particularly in PL (Table 5). Likewise, cells from control fish presented with 19:2n-6 contained 17:2, 21:2, and 23:2, while those from fish fed olive oil contained in addition 21:3 and 21:4. The levels of C_{21} and C_{23} OCLUFA were higher in

TABLE 3
Fatty Acid Composition (wt%) of Total Lipids (TL), Triacylglycerols (TAG), and Polar Lipids (PL) from Hepatocytes of Trout Fed Control and Olive Oil Diets[a]

	Hepatocyte TL		Hepatocyte TAG		Hepatocyte PL	
Fatty acids	Control	Olive oil	Control	Olive oil	Control	Olive oil
14:0	1.9 ± 0.2	1.3 ± 0.4	3.9 ± 0.7	1.9 ± 0.4*	2.1 ± 0.3	0.7 ± 0.3*
15:0	0.5 ± 0.1	trace	0.4 ± 0.1	trace	trace	trace*
16:0	18.3 ± 0.1	16.2 ± 0.1*	14.3 ± 0.5	12.9 ± 0.5	20.2 ± 0.3	16.7 ± 0.8*
16:1n-9 + n-7	2.8 ± 0.3	4.1 ± 1.0	7.8 ± 0.2	6.2 ± 1.0	3.1 ± 0.4	2.6 ± 0.6
16:2	0.4 ± 0.0	—*	0.5 ± 0.1	—	0.4 ± 0.0	—*
16:3	—	trace	0.4 ± 0.0	trace*	trace	trace*
18:0	5.2 ± 0.6	6.2 ± 0.5	3.1 ± 0.4	4.6 ± 0.2*	5.6 ± 1.1	6.1 ± 0.4
18:1n-9 + n-7[b]	10.9 ± 0.1	27.0 ± 4.2*	24.4 ± 0.7	50.6 ± 5.6*	9.4 ± 0.2	21.1 ± 3.0*
18:1n-5	0.5 ± 0.1	trace	0.6 ± 0.1	trace*	0.5 ± 0.0	trace*
18:2n-6	2.7 ± 0.1	3.5 ± 0.3*	4.8 ± 0.6	2.9 ± 1.2	2.2 ± 0.2	3.8 ± 0.1*
18:3n-3	0.5 ± 0.0	trace*	1.0 ± 0.1	trace*	0.4 ± 0.0	trace
18:4n-3	0.4 ± 0.1	trace*	0.8 ± 0.1	trace*	trace	trace
20:1n-11 + n-9	3.8 ± 0.0	3.9 ± 0.9	6.2 ± 0.2	5.8 ± 1.3	3.5 ± 0.0	3.6 ± 0.9
20:2n-9	—	0.8 ± 0.3	—	1.1 ± 0.2*	—	0.7 ± 0.3
20:2n-6	0.6 ± 0.0	0.5 ± 0.2	0.7 ± 0.1	0.6 ± 0.3	0.6 ± 0.0	0.6 ± 0.1
20:3n-9	—	2.0 ± 0.9	—	1.0 ± 0.1*	—	2.4 ± 1.0
20:3n-6	0.4 ± 0.0	2.3 ± 1.0*	trace	0.9 ± 0.4	0.5 ± 0.0	2.9 ± 0.8*
20:4n-6	2.5 ± 0.3	6.4 ± 1.9*	0.9 ± 0.2	1.8 ± 1.0	2.8 ± 0.2	7.7 ± 1.2*
20:4n-3	0.8 ± 0.1	trace*	1.1 ± 0.1	trace*	0.6 ± 0.0	trace*
20:5n-3	6.4 ± 0.4	1.4 ± 0.6*	5.3 ± 1.4	0.7 ± 0.3*	6.3 ± 0.7	1.7 ± 0.9*
22:1n-11 + n-9	0.7 ± 0.2	trace*	2.9 ± 0.1	0.6 ± 0.1*	trace	trace
22:4n-6	—	0.5 ± 0.2	—	trace	—	0.6 ± 0.1*
22:5n-6	—	2.4 ± 1.3	—	0.6 ± 0.3	trace	2.9 ± 1.2*
22:5n-3	1.2 ± 0.1	0.6 ± 0.1*	1.5 ± 0.2	0.4 ± 0.1*	1.1 ± 0.1	0.7 ± 0.2
22:6n-3	39.4 ± 0.3	19.3 ± 4.0*	18.6 ± 0.1	5.2 ± 1.5*	39.1 ± 1.7	23.8 ± 3.3*
24:6n-3	—	—	0.4 ± 0.0	—*	—	—
Total saturates	25.9 ± 0.7	23.7 ± 1.0	21.7 ± 1.8	19.5 ± 1.0	28.2 ± 1.1	23.6 ± 1.0*
Total monoenes	18.7 ± 0.8	35.5 ± 5.9*	41.9 ± 0.4	63.5 ± 5.3*	16.8 ± 0.6	27.6 ± 4.4*
Total PUFA	55.2 ± 0.3	40.4 ± 6.7	36.1 ± 1.3	16.3 ± 4.9*	54.7 ± 1.9	48.3 ± 3.3
Total n-6	6.1 ± 0.4	15.6 ± 2.2*	6.5 ± 0.3	7.0 ± 3.3	6.3 ± 0.5	18.4 ± 2.5*
Total n-3	48.7 ± 0.5	21.7 ± 4.2*	28.7 ± 1.8	6.9 ± 1.5*	47.7 ± 2.4	26.5 ± 4.4*
n-6/n-3	0.1 ± 0.0	0.7 ± 0.2*	0.2 ± 0.0	1.0 ± 0.2*	0.1 ± 0.0	0.7 ± 0.2*
Unidentified	0.2 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.7 ± 0.4	0.3 ± 0.2	0.6 ± 0.2

[a]Data are means ± SD (n = 3 fish); —, not detected; trace, fatty acid values <0.4%. Pairs of values within a given row were compared by a Student's t-test and those that were significantly different ($P < 0.05$) are shown (*). [b]Mainly 18:1n-9 isomer; PUFA, polyunsaturated fatty acids.

lipids of hepatocytes prepared from OO fed fish and incubated with 19:3n-3 than in hepatocytes from control fish presented with the same substrate. In particular, the levels of 21:4 and 21:5 were higher in the cells from the fish fed the OO diet. For all three C_{19} substrates, the lipids of cells from fish fed OO contained in general more OCLUFA products than cells from control fish. With hepatocytes from both dietary groups of fish, the proportion of incorporated C_{19} substrate

TABLE 4
Incorporation of Odd-Chain Length Unsaturated Fatty Acids (nmol/mg protein) into Total Lipids, Triacylglycerols, and Polar Lipids of Trout Hepatocytes[a]

	Total lipids		Triacylglycerols		Polar lipids	
Fatty acids	Control	Olive oil	Control	Olive oil	Control	Olive oil
19:1n-9	7.5 ± 2.3^{a,b,c}	10.2 ± 3.5^{c,d}	4.7 ± 1.4^{a,b}	5.1 ± 2.4^{a,b}	2.7 ± 0.6^{b}	3.4 ± 0.9^{a,b}
19:2n-6	5.9 ± 0.9^{a,b,c}	8.5 ± 2.3^{b,c,d}	3.6 ± 0.3^{a,b}	5.1 ± 2.4^{a,b}	2.0 ± 0.5^{a,b}	3.2 ± 0.5^{a,b,*}
19:3n-3	9.4 ± 2.6^{c}	11.5 ± 1.5^{d}	7.3 ± 2.6^{b}	6.1 ± 3.0^{b}	2.7 ± 1.5^{b}	4.0 ± 0.5^{b}
21:2n-6	5.0 ± 1.0^{a,b}	4.9 ± 0.7^{a,b,c}	3.6 ± 1.9^{a,b}	3.4 ± 0.9^{a,b}	0.7 ± 0.4^{a}	1.7 ± 0.5^{a}
21:3n-6	6.7 ± 0.3^{a,b,c}	3.3 ± 0.8^{a,b,*}	4.1 ± 2.2^{a,b}	1.7 ± 0.3^{a,b}	2.0 ± 1.0^{a,b}	1.7 ± 0.9^{a}
21:4n-6	8.1 ± 1.2^{b,c}	9.9 ± 1.3^{c,d}	5.4 ± 2.2^{a,b}	5.5 ± 3.3^{b}	1.7 ± 0.5^{a,b}	4.2 ± 0.9^{b,*}
21:3n-3	3.2 ± 0.7^{a}	2.2 ± 0.7^{a}	2.2 ± 0.7^{a}	0.8 ± 0.2^{a,*}	0.6 ± 0.3^{a}	1.6 ± 0.5^{a,*}
21:5n-3	9.6 ± 2.0^{c}	12.4 ± 2.9^{d}	7.2 ± 2.3^{b}	6.2 ± 3.1^{b}	1.6 ± 0.5^{a,b}	4.9 ± 0.5^{b,*}

[a]Data are means ± SD (n = 3 fish). Pairs of means within a given row were compared by a Student's t-test and those that were significantly different ($P < 0.05$) are shown (*). Means within a given column were compared by a one-way analysis of variance followed by Tukey's multiple comparison test; values not bearing the same superscript letter are significantly different ($P < 0.05$).

TABLE 5
Recovery (nmol/mg protein) of Fatty Acids Derived from C_{19} Fatty Acid Substrates in Total Lipids (TL), Triacylglycerols (TAG), and Polar Lipids (PL) of Trout Hepatocytes[a]

	TL		TAG		PL	
	C	OO	C	OO	C	OO
19:1n-9						
17:1	1.8	2.8	0.7	1.0	1.6	2.1
21:1	0.7	1.3	0.5	0.7	—	0.2
23:1	0.3	0.7	0.2	0.4	—	trace
Total	2.8	4.8	1.4	2.1	1.6	2.3
19:2 n-6						
17:2	1.1	1.5	0.5	0.9	0.6	1.0
21:2	0.5	2.0	0.4	1.3	0.1	0.5
21:3	—	0.5	—	0.3	—	0.1
21:4	—	0.3	—	0.1	—	0.1
23:2	0.1	0.5	0.1	0.3	—	—
Total	1.7	4.8	1.0	2.9	0.7	1.7
19:3n-3						
17:3	0.8	1.2	0.3	0.4	0.1	0.6
19:4	—	0.6	—	0.4	—	0.2
21:3	0.9	1.3	0.7	0.7	0.1	0.5
21:4	0.5	4.0	0.5	1.7	—	1.7
21:5	0.4	1.4	0.6	0.6	—	0.7
23:3	—	—	trace	0.1	—	—
23:4	—	—	—	0.1	—	—
23:5	—	—	—	0.1	—	—
Total	2.6	8.5	2.1	4.1	0.2	3.7

[a]—, not detected; trace, value <0.05. C, control diet; OO, olive oil diet.

TABLE 6
Recovery (nmol/mg protein) of Fatty Acids Derived from C_{21}, n-6 Series Fatty Acid Substrates in Total Lipids (TL), Triacylglycerols (TAG), and Polar Lipids (PL) of Trout Hepatocytes[a]

	TL		TAG		PL	
	C	OO	C	OO	C	OO
21:2n-6						
17:2	2.0	1.4	1.1	1.1	1.1	0.3
19:2	1.9	2.4	1.1	1.5	0.6	1.3
19:3	—	0.9	—	0.3	—	0.3
21:3	—	1.1	0.1	0.5	—	0.7
21:4	—	0.8	—	trace	—	0.8
23:2	0.8	0.9	0.4	0.6	—	0.2
Total	4.7	7.5	2.7	4.0	1.7	3.6
21:3n-6						
17:3	—	—	0.1	0.1	—	—
19:2	—	trace	—	—	—	0.4
19:3	0.8	0.3	0.2	0.1	0.3	0.2
19:4	—	0.4	—	0.1	—	0.7
21:2	—	—	—	—	—	trace
21:4	1.5	5.5	0.8	2.9	0.4	1.9
23:3	—	—	—	trace	—	—
23:4	—	0.3	—	0.1	—	—
Total	2.3	6.5	1.1	3.3	0.7	3.2
21:4n-6						
17:4	—	0.3	—	0.2	—	—
19:4	0.5	0.7	0.2	0.3	0.3	0.4
21:3	—	—	—	0.1	—	—
23:4	0.3	0.6	0.1	0.3	—	0.1
Total	0.8	1.6	0.3	0.9	0.3	0.5

[a]Footnote as in Table 5.

subjected to chain-shortening decreased in the order 19:1n-9 > 19:2n-6 > 19:3n-3 while the proportion of 19:3n-3 elongated and desaturated was greater than those of 19:2n-6 or 19:1n-9, particularly in hepatocytes from OO- fed fish. The chain-shortened products of 19:1n-9 and 19:2n-6 were notably more prominent in PL than in TAG. When incubated with the C_{21}n-6 substrates, 21:2, 21:3, and 21:4, cells prepared from the livers of fish fed the OO diet generated more OCLUFA products than cells from the livers of control fish (Table 6). Thus, in the TL of hepatocytes from control fish incubated with 21:2n-6, 19:2 and 17:2 were the main products along with a smaller amount of 23:2, whereas in cells from fish fed OO, 21:3 and 21:4 were also present in significant amounts. It was notable that 21:4 was the major OCLUFA in the PL fraction of the latter cells. Likewise, 21:4 was abundant in the lipids of cells presented with 21:3n-6, particularly those of trout fed OO which also contained small amounts of 19:4. Although the lipids of cells incubated with 21:4n-6 contained small amounts of 19:4, 17:4, and 23:4, the substrate itself was always the major odd-chain length fatty acid and no OCLUFA with more than four double bonds were detected.

The profiles of OCLUFA present in hepatocytes incubated with 21:3n-3 and 21:5n-3 are shown in Table 7. The lipids of cells from both dietary groups produced 19:3, 17:3, 21:4, and 21:5 when presented with 21:3n-3. The amounts of 21:5 and particularly 21:4 were higher in cells from fish maintained on OO with the highest levels being recovered in PL. When 21:5n-3 was the substrate, 19:5 and 23:5 were recovered in the lipids of cells from both the control and the OO-treated fish. In addition, hepatocytes from fish fed the OO diet contained 23:6 in both TL and PL and small amounts of 25:5 and 25:6 in TAG.

For the three n-3 OCLUFA used in the present study, the amounts of incorporated substrate and products recovered in hepatocyte total lipid were 19:3 > 21:5 > 21:3 for both the control and the n-3-deficient cells.

TABLE 7
Recovery (nmol/mg protein) of Fatty Acids Derived from C_{21} n-3 Series Fatty Acid Substrates in Total Lipids (TL), Triacylglycerols (TAG), and Polar Lipids (PL) of Trout Hepatocytes[a]

	TL		TAG		PL	
	C	OO	C	OO	C	OO
21:3n-3						
17:3	0.3	0.8	0.1	0.1	trace	0.6
19:3	1.0	1.0	0.5	0.4	0.4	1.0
19:4	—	0.8	0.2	0.1	—	0.4
21:4	1.2	5.6	0.7	1.4	—	4.0
21:5	0.1	0.6	trace	trace	—	1.0
23:3	0.2	—	0.1	—	—	—
Total	2.8	8.8	1.6	2.0	0.4	7.0
21:5n-3						
19:5	0.2	0.2	0.1	0.1	—	0.2
23:5	0.5	0.6	0.1	0.2	0.1	0.2
23:6	—	0.6	—	—	—	0.6
25:5	—	—	—	0.1	—	—
25:6	—	—	—	trace	—	—
Total	0.7	1.4	0.2	0.4	0.1	1.0

[a]Footnote as in Table 5.

DISCUSSION

The observed differences in fatty acid composition between hepatocytes from fish of the two dietary groups are all consistent with the known enhanced use of 18:1n-9 and 18:2n-6 as substrates for desaturation and elongation in the absence of dietary n-3 PUFA. It has been shown previously that the n-3 PUFA-deficient hepatocytes from trout fed OO exhibit an increased rate of conversion of 18:3n-3 to 22:6n-3 *in vitro* when presented with the former PUFA and offer a good system for the study of fatty acid desaturation and elongation (3). In view of their higher lipid content, hepatocytes from fish fed OO might have been expected to contain a higher proportion of TAG in their lipid than cells from control fish. The fact that the lipid class compositions of the two groups of cells were very similar may be due to the 18:1n-9-rich TAG of hepatocytes from fish fed OO being underestimated during staining and densitometry (23–25).

The occurrence of appreciable quantities of odd-chain length PUFA is not common in animal tissues although very small amounts of 21:5 have been reported in almost every phylum of marine animals including fish (10,11) and seals (12). Thus, in the present study, any odd-chain PUFA recovered in hepatocyte lipids could be assumed to have been derived from the OCLUFA included in the incubation medium. The recovery of significant amounts of all eight substrates examined in cellular lipid indicates that trout hepatocytes can readily assimilate long-chain unsaturated fatty acids having an odd number of carbons. The rates of uptake of 19:3n-3 by hepatocytes from fish fed the OO diet into TAG and PL and its overall rate of conversion to other more unsaturated derivatives are very similar to those found previously for ^{14}C-18:3n-3 with hepatocytes from trout maintained on a similar diet (3).

The range of odd-chain length fatty acids observed in hepatocyte lipids indicates that the assimilated OCLUFA were utilized as substrates for chain-shortening as well as desaturation and elongation. It was notable that of the eight substrates examined, 21:2n-6 was subjected most to chain-shortening by hepatocytes from trout of both dietary groups, followed by 21:3n-3 and 19:1n-9. The even number equivalents of 21:2n-6 and 21:3n-3, 20:2n-6, and 20:3n-3, respectively, are not major components of natural lipids, and it might be expected that these unusual fatty acids are chain-shortened in a manner similar to the induced peroxisomal oxidation which occurs in rat hepatocytes presented with high levels of 22:1n-9 and other long-chain monounsaturated fatty acids (26). In contrast, the C_{18} equivalent of 19:1n-9, 18:1n-9, occurs in naturally high levels in lipids and is not an inducer of peroxisomal oxidation. The presence of the extra carbon, however, may be sufficient to render 19:1n-9 a substrate for oxidation. Nevertheless, from the results obtained in the present study, it is not possible to conclude whether the oxidation of OCLUFA occurred in peroxisomes or mitochondria. Chain-shortening of fatty acids which are poorly oxidized by mitochondria has been considered the main physiological role of peroxisomal β-oxidation (27). Since OCLUFA are not common substrates in cell metabolism, it is possible that their oxidation is taking place in the peroxisomes, organelles which seem to have in fish a broader range of substrate selectivity than mitochondria (28). The oxidation of some C_{20}, C_{22}, and C_{24} members of the different fatty acid series has been reported to be initiated in peroxisomes rather than mitochondria (29,30), and it is known that PUFA are relatively poor substrates for mitochondrial β-oxidation in fish (31,32). With the exception of 19:1n-9, all the substrates used were PUFA and/or long-chain fatty acids, which brings us to speculate that peroxisomes are more likely to be responsible for the chain-shortening of these fatty acids.

The pattern of products observed is consistent with chain-shortening proceeding *via* the removal of two-carbon units, i.e. β-oxidation. On the basis of the recovery of small amounts of radioactivity from [U-^{14}C]-acetate in a whole range of odd chain length PUFA from 17:2 to 29:5, Aveldaño *et al.* (13) suggested that α-oxidation is involved in the formation of PUFA in rat testes. Although the removal of a single carbon by α-oxidation cannot be completely ruled out in the present study, no noticeable increases in the levels of 20:2n-6 and 20:3n-3 in lipids were observed in cells presented with 21:2n-6 and 21:3n-3, respectively, as might be expected if α-oxidation occurred actively.

The recovery of larger amounts of the chain-shortened products from 19:1n-9, 21:2n-6, and 21:3n-3 in PL of cells from both dietary groups suggests that the shorter-chain products are more acceptable for PL structure than the original substrates. In contrast, the original substrates can be readily esterified into TAG in keeping with its role as a store of fatty acids not required for PL synthesis. Since the proportions of fatty acids recovered as chain-shortened products were similar in TL of both groups of cells, it appears that the rate of chain-shortening is not greatly influenced by dietary lipid composition. However, there was a decrease in the incorporation of the chain-shortened fatty acids from 21:2n-6 and 21:3n-3 in the PL of cells from the OO-fed fish, together with an increment of their elongation–desaturation products in the same lipid fraction. This implies that chain-shortening may act in conjunction with elongation–desaturation to generate the desired PUFA.

The more extensive desaturation and elongation of n-6 and n-3 substrates observed with hepatocytes from trout maintained on OO than from control fish is in keeping with the former cells being deficient in PUFA, particularly n-3 PUFA, and having therefore an increased need to synthesize long-chain PUFA from shorter, less unsaturated PUFA, as has been demonstrated previously with radiolabeled substrates (3).

The extensive desaturation of 21:3n-6 to 21:4n-6 and of 21:3n-3 to 21:4n-3 and then to 21:5n-3 was particularly notable in the n-3 PUFA-deficient hepatocytes. The higher recovery of the resultant 21:4n-3 than 21:4n-6 in the PL of these n-3-deficient cells indicates that the substrate preference of the phospholipid esterification system for n-3 PUFA observed in fish (2,3) holds even for odd-chain PUFA.

It is difficult to conclude exactly how the 21:5n-3 was formed from 21:3n-3 since, although the action of a Δ6 desaturase on 21:3n-3 would produce 6,12,15,18-21:4, the subsequent direct conversion of this fatty acid to 21:5n-3 by Δ5 desaturase would not be feasible owing to the presence of the double bond at the C_6 position. Since the hepatocytes were active in chain-shortening it is possible that the 21:3n-3 substrate was first shortened to 10,13,16-19:3 which was desaturated to 6,10,13,16-19:4 followed by elongation to 8,12,15,18-21:4. This form of 21:4 would be a substrate for Δ5 desaturase to produce 5,8,12,15,18-21:5. Similar pathways may exist for the conversion of 21:2n-6 to 21:4n-6. The high levels of chain-shortened OCLUFA products found in the lipids of the trout hepatocytes suggest that such a pathway involving chain-shortening and Δ6 and Δ5 desaturases may operate. Consequently, the involvement of a Δ8 desaturase in the direct conversion of 21:3n-3 and 21:2n-6 to 21:5n-3 and 21:4n-6, respectively, is likely to be of minor importance, in keeping with results obtained recently by Schenck *et al.* (33) in the mouse liver.

The more extensive recovery of nonmodified 21:5n-3 and 21:4n-6 in hepatocyte lipids in comparison with the other substrates examined is in keeping with these structures being suitable for incorporation directly into cellular lipids without further elongation and desaturation or chain-shortening. Arachidonic acid, 20:4n-6, has been reported to be oxidized at a negligible rate in peroxisomes of rainbow trout (32). Although PUFA are substrates for peroxisomal β-oxidation in rodents (34,35) and at least one species of fish (28), a poor β-oxidation rate had been reported for 20:4n-6 and 20:5n-3 by Hovik and Osmundsen (34). While the low rate of chain-shortening of 21:4n-6 and 21:5n-3 observed in the present study may have been attributable to a discrimination against them by the oxidation system in control hepatocytes, such an explanation is unlikely in the cells from fish fed OO, since the virtual absence of long-chain PUFA in the hepatocyte lipids would favor the esterification of the substrates into cellular lipids rather than removal by chain-shortening.

In conclusion, the results presented here demonstrate that the pathways of PUFA conversions in trout hepatocytes can utilize odd-chain unsaturated fatty acids and that these substrates are subjected to chain-shortening as well as desaturation and elongation. The importance of chain-shortening in the formation of 22:6n-3 *via* a Δ4 desaturase-independent pathway has been firmly established in rat (4) and trout (3) hepatocytes, and it has also been demonstrated that 22:5n-6 arises from the partial oxidation of 24:5n-6 in rat liver (36). The occurrence of both 25:5n-3 and 25:6n-3 in hepatocytes incubated with 21:5n-3 is consistent with the production of 23:6n-3 by these cells involving C_{25} intermediates. The present results suggest that chain-shortening may be an integral part of PUFA metabolism. Further studies to determine the position of the double bonds in the desaturation products of the OCLUFA are necessary to characterize the positional specificity of the desaturases present in trout hepatocytes.

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Lipid Content and Fatty Acid Composition of 11 Species of Queensland (Australia) Fish

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ABSTRACT: The fatty acid composition of 11 species of fish caught off the northeast coast of Australia was determined. No fatty acid profiles have been previously published for fish from this area nor for nine of these species. Although the percentage of polyunsaturated fatty acid (PUFA) was the same as the calculated average for Australian fish (42.3%), the percentage of n-3 fatty acids was lower (24.4 ± 5.4% vs. 30.7 ± 10.1%) and the n-6 fatty acids higher (16.5 ± 4.5% vs. 11.2 ± 5.9%), $P < 0.001$ in each case. The major n-3 PUFA were docosahexaenoic (15.6 ± 6.3%) and eicosapentaenoic acid (4.3 ± 1.1%) while the major n-6 PUFA were arachidonic (8.3 ± 3.2%) and n-6 docosatetraenoic acid (3.1 ± 1.3%). The second-most abundant class of fatty acid was the saturates (31.6 ± 3.5%) while the monounsaturates accounted for 17.4 ± 4.3% of the total fatty acids. The monounsaturate with the highest concentration was octadecenoic acid (11.8 ± 2.6%). There was a positive correlation between the total lipid content and saturated and monounsaturated fatty acids ($r = 0.675$ and 0.567, respectively) and a negative correlation between the total lipid content and PUFA ($r = 0.774$).

Lipids 32, 621–625 (1997).

Long-chain polyunsaturated fatty acids (PUFA) of fish (20:5 n-3 eicosapentaenoic acid and 22:6 n-3 docosahexaenoic acid) have been reported to affect vascular function (1) which contributes to a decrease in the overall risk of cardiovascular disease. Incorporation of these long-chain PUFA into cardiovascular tissue membranes occurs readily, mainly at the expense of PUFA of the n-6 type (18:2 and 20:4). The benefits of fish oil fatty acids against thrombogenic cardiovascular risk factors such as platelet/vessel wall interactions are achieved *via* a favorable eicosanoid profile. For example, compared to the prostaglandins and thromboxanes of the 2-series (PGI_2 and TxA_2) which derive from membrane arachidonic acid (n-6, 20:4), n-3 PUFA result in the production of PGI_3 and TxA_3 (2,3). This 3-series of eicosanoids derived from n-3 PUFA have different thrombogenic potencies in comparison to those derived from n-6 PUFA. Thus, PGI_3 is equipotent to PGI_2 in antithrombotic actions while TxA_3 possesses only weak biological activity, thus the risk for ischemic heart disease is reduced (4). Studies have shown that dietary fish oil reverses impairment of endothelium-dependent relaxation (5), inhibits platelet activation (6), reduces monocyte attachment to arterial endothelium, and suppresses release of toxic, mitogenic, and prothrombotic agents (7). There has also been one Australian study showing that eating fatty fish improved hemostatic factors in humans (8).

Very little has been published on the fatty acid composition of Australian fish. Marine fish lipids are a rich source of n-3 fatty acids, and in addition it has been observed that many Australian fish are relatively rich sources of n-6 PUFA when compared with Northern Hemisphere fish (9). Collection areas for Australian fish for which fatty acid data have been published are the Sydney area (10–12), Northwest coast (13), Adelaide (14), Northwest shelf (15,16), and Victoria (17). These publications have been reviewed and the fatty acid data collated (9,18). We present the fatty acid composition of fish from another area of Australian waters, off the Queensland coast at a latitude of about 23°S.

MATERIALS AND METHODS

One hundred eleven fish representing 11 species and 6 families were caught off the reef at Heron Island. The fish were caught with hand-lines in January or June with a mixture of species on each occasion. The freshly caught fish were scaled and samples of about 3 g were dissected from a well-defined and constant part of the dorsal muscle between the dorsal fin and the head. Skin was included as this is usually eaten. The sampling was always done by the same person and was highly reproducible. The samples were added to preweighed vials containing 12 mL of chloroform/methanol (2:1) and the vials reweighed. The length and species of each fish were recorded. All fish were above the minimum legal size which is 23 cm for yellow fin bream; 30 cm for collared sea bream, sweetlip emperor, venus tuskfish, parrot fish and hussar; 35 cm for chickenwire cod, coral trout, red emperor, and yellow stripey. All species are commonly available in fish markets in Australia. Sweetlip emperor, red emperor, and coral trout are ex-

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Abbreviations: MUFA, monounsaturated fatty acids; PGI_3, prostaglandin I_3; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TxA_3, thromboxane A_3.

cellent eating and are the most highly sought, collared sea bream, yellowtail bream, yellow stripey, and venus tuskfish are well accepted and readily obtainable in the fish markets. Chickenwire cod, longfin rock cod, parrot fish, and hussar are less popular in fish markets but still available and reasonably good eating.

Samples were cut into small pieces, returned to the chloroform/methanol solution, and extracted according to the method of Folch *et al.* (19). Total lipids were determined gravimetrically. Fatty acid composition was determined on the lipid extracts after methylation with 1% sulfuric acid in dry methanol (20). The fatty acid methyl esters were extracted with petroleum ether (B.P. 40–60°C) and contaminants removed by chromatography on florisil (Sigma Chemical Co., St. Louis, MO). The eluate was dried under nitrogen and the samples were taken up in isooctane. An aliquot was injected onto a gas chromatographic column of vitreous silica (30 m × 0.53 mm) coated with cross-linked free fatty acid phase in a Hewlett-Packard 5711A gas chromatograph (Hewlett-Packard, Avondale, PA) (21). Fatty acids were identified by comparison with authentic standards supplied by Nu-Chek-Prep (Elysian, MN) and a sample of fish oil (l8919BB) which had been analyzed by U.S. Department of Commerce Southeast Fisheries Science Center. Correction factors were used to convert area percentage to weight percentage.

RESULTS AND DISCUSSION

Table 1 shows the common and scientific names, the number analyzed, and the mean and range of total lipid content of the fish from this study. Generally the fish were lean: 74 had a total lipid content of less than 2% and only 9 of the 111 fish had a total lipid content greater than 5%. The species with the largest variation was collared sea bream with 17 of the 31 fish having less than 2% total lipids and 6 fish more than 5%.

Twenty-four fatty acids were identified in all samples of fish and Table 2 shows mean fatty acid profiles for these fatty acids in the 11 species. As well as these fatty acids, the column was able to resolve some minor unidentified fatty acids and the dimethylacetals of stearic and palmitic acids. Table 3 shows the total n-6 fatty acids, total n-3 fatty acids, the n-6/n-3 ratios, the totals of saturated (SFA), monounsaturated (MUFA), and PUFA, and the polyunsaturated-to-saturated ratio for each species.

The PUFA were the most abundant group of fatty acids for all species, accounting for 42.3 ± 6.9% of the total fatty acids. For the 111 samples representing the 11 species, the n-3 series PUFA were present as 24.4 ± 5.4% of the total fatty acids, and the n-6 series PUFA were present as 16.5 ± 4.5% of the total fatty acids, whereas Brown *et al.* (9) calculated means of 30.7 ± 10.1% and 11.2 ± 5.9% for n-3 PUFA and n-6 PUFA, respectively, for Australian fish. While the mean percentage of total PUFA is the same for Queensland (this study) and Australian fish (9), 42.3%, the percentage of n-6 PUFA is higher for the Queensland fish. Sinclair *et al.* (22) showed that the relative n-6 PUFA content of fish increased as the sampling point moved closer to the equator and suggested that the supply of n-6 PUFA in the marine food chain increased in tropical waters.

Gibson (14) and Sinclair *et al.* (13) have emphasized that fish from both southern Australian waters and from the northwest coast of Australia are rich in arachidonic acid. Brown *et al.* (9) have calculated that arachidonic acid on average constituted 6.6% of the total fatty acids in Australian fish. Arachidonic acid (20:4) was the major n-6 PUFA in the Queensland fish in our study (8.3 ± 3.2%) followed by n-6 docosatetraenoic acid (22:4) (3.1 ± 1.3%), n-6 docosapentaenoic acid (22:5) (1.9 ± 0.7%), and linoleic acid (18:2) (1.7 ± 0.8 %).

Most abundant of the n-3 PUFA was 22:6 (docosahexaenoic acid) (15.6 ± 6.3%), while 20:5 (eicosapentaenoic acid) and n-3 22:5 (docosapentaenoic acid) were also present in important proportions (4.3 ± 1.1% and 3.5 ± 0.9%, respectively).

The second-most abundant group of fatty acids was the SFA which constituted 31.6 ± 3.5% of the total fatty acids. The highest concentrations of SFA were palmitic acid (16:0)

TABLE 1
Total Lipid Content of Fish Samples

Common name	Scientific name	Number analyzed	Lipids (g/100 g) (wet weight basis)	
			Mean	Range
Collared sea bream (iodine bream)	*Gymnocranius audleyi*	31	2.7	0.2–10.8
Sweetlip emperor	*Lethrinus miniatus*	18	2.1	0.4–7.1
Venus tuskfish	*Choerodon venustus*	13	1.8	0.3–7.5
Chickenwire cod	*Epinephelus merra*	11	1.6	0.6–3.0
Yellowtail bream	*Acanthopagrus australis*	9	0.8	0.4–1.2
Longfin rock cod	*E. quoyanus*	8	0.5	0.2–0.8
Coral trout	*Plectroponus leopardus*	7	2.4	0.5–4.8
Red emperor	*Lutjanus sebae*	5	1.6	0.6–4.6
Parrot fish	*C. albigera*	4	1.3	0.4–2.0
Hussar	*Lu. adetii*	3	4.9	4.1–5.7
Yellow stripey (Spanish flag)	*Lu. carporonatus*	2	1.3	0.7–1.9

TABLE 2
Fatty Acid Analysis of Fish Samples (wt% of total fatty acids)

	Number		14:0	15:0	16:0	16:1	16:2	16:3	17:0	18:0	18:1	18:2n-6	18:3n-6	18:3n-3	18:4n-3
Collared sea bream	31	Mean	2.0	1.1	19.4	4.8	0.5	1.1	1.1	8.2	11.4	1.5	0.5	0.5	0.3
		SD	0.9	0.3	1.6	1.8	0.1	0.4	0.4	0.8	2.5	0.3	0.1	0.2	0.1
Sweetlip emperor	18	Mean	2.2	0.9	20.7	4.1	0.4	0.9	1.1	8.5	13.1	2.0	0.6	0.9	0.3
		SD	0.8	0.5	2.7	1.3	0.1	0.3	0.4	0.7	2.7	0.5	0.1	0.3	0.1
Venus tuskfish	13	Mean	2.6	1.1	19.4	3.4	0.6	1.0	1.0	7.7	11.3	2.0	0.5	0.5	0.4
		SD	1.7	0.4	2.1	1.6	0.4	0.3	0.3	0.8	2.0	1.1	0.1	0.2	0.4
Chickenwire cod	11	Mean	1.5	0.7	20.5	3.6	0.5	1.1	0.8	8.7	12.5	2.0	0.5	1.1	0.4
		SD	0.7	0.4	1.7	1.2	0.1	0.4	0.1	0.8	1.9	0.6	0.1	0.4	0.4
Yellowtail bream	9	Mean	1.3	0.9	17.2	3.3	0.5	0.8	1.3	8.9	9.5	1.2	0.6	0.4	0.2
		SD	0.7	0.4	1.4	1.3	0.0	0.2	0.5	0.7	2.2	0.1	0.2	0.2	0.1
Longfin rock cod	8	Mean	1.6	0.5	19.4	3.4	0.7	0.6	0.9	8.7	11.5	2.4	0.5	0.7	0.3
		SD	1.0	0.2	3.0	1.2	0.1	0.3	0.2	0.8	1.5	1.9	0.1	0.3	0.1
Coral trout	7	Mean	5.4	0.9	22.8	6.6	0.7	0.6	1.0	7.3	12.2	1.3	0.5	0.9	0.5
		SD	2.2	0.1	2.1	1.7	0.1	0.1	0.1	1.0	2.3	0.2	0.2	0.5	0.1
Red emperor	5	Mean	1.8	0.9	20.5	3.7	0.4	0.8	1.0	8.4	13.3	1.4	0.4	0.7	0.4
		SD	1.3	0.5	3.0	2.2	0.2	0.3	0.4	0.7	4.9	0.3	0.1	0.3	0.3
Parrot fish	4	Mean	1.8	0.8	18.3	2.6	0.5	0.6	1.0	8.4	10.2	1.4	0.4	0.6	0.3
		SD	1.4	0.4	2.5	1.2	0.1	0.3	0.3	1.0	2.1	0.3	0.1	0.2	0.2
Hussar	3	Mean	3.2	0.8	22.8	8.2	0.7	1.0	0.7	6.3	12.8	1.1	0.4	0.5	0.4
		SD	0.2	0.4	1.5	1.6	0.1	0.1	0.1	0.1	1.3	0.1	0.1	0.2	0.1
Yellow stripey	2	Mean	2.5	1.1	22.8	5.4	0.7	0.7	0.8	7.2	14.0	1.3	0.7	0.9	0.7
		SD	0.3	0.6	1.6	0.9	0.2	0.1	0.2	0.5	3.4	0.1	0.4	0.2	0.2

	Number		20:0	20:1	20:2n-6	20:3n-6	20:4n-6	20:5n-3	22:1	22:4n-6	22:5n-6	22:5n-3	22:6n-3
Collared sea bream	31	Mean	0.4	1.1	0.5	0.5	8.4	4.7	0.7	4.2	1.9	3.8	13.2
		SD	0.1	0.8	0.1	0.1	2.0	0.8	0.1	0.7	0.7	0.7	5.3
Sweetlip emperor	18	Mean	0.4	0.8	0.6	0.7	8.9	4.4	0.7	3.0	1.7	3.4	12.6
		SD	0.2	0.5	0.2	0.2	2.4	1.1	0.2	0.7	0.8	0.6	6.6
Venus tuskfish	13	Mean	0.3	0.6	0.6	0.4	9.9	4.3	0.5	2.3	2.1	3.1	17.0
		SD	0.2	0.3	0.2	0.1	5.2	0.9	0.1	0.7	0.7	1.4	5.6
Chickenwire cod	11	Mean	0.3	0.6	0.5	0.8	9.9	3.9	0.4	2.9	1.8	3.9	13.7
		SD	0.1	0.3	0.2	0.2	2.7	0.9	0.1	0.6	0.5	0.5	5.8
Yellowtail bream	9	Mean	0.3	0.5	0.6	0.5	10.1	5.0	0.7	5.0	2.1	4.8	14.5
		SD	0.3	0.2	0.2	0.1	3.1	1.8	0.1	0.5	0.6	0.9	4.8
Longfin rock cod	8	Mean	0.3	0.6	0.5	0.6	7.9	3.1	0.4	2.7	2.4	3.0	19.9
		SD	0.1	0.2	0.1	0.1	2.1	0.9	0.1	0.5	0.6	0.3	3.1
Coral trout	7	Mean	0.3	0.7	0.3	0.4	3.4	3.5	0.1	1.2	1.8	2.8	20.0
		SD	0.1	0.3	0.1	0.1	1.7	0.7	0.1	0.7	0.6	0.7	3.5
Red emperor	5	Mean	0.3	0.6	0.5	0.4	6.9	4.3	0.3	1.9	1.6	3.1	20.2
		SD	0.1	0.6	0.1	0.2	0.8	1.4	0.1	0.3	0.7	0.4	11.4
Parrot fish	4	Mean	0.3	0.4	0.6	0.5	8.2	4.5	0.6	2.2	2.4	3.4	21.1
		SD	0.2	0.1	0.2	0.1	1.9	1.6	0.3	0.4	0.9	0.3	6.5
Hussar	3	Mean	0.3	0.6	0.3	0.2	2.8	3.7	0.5	1.0	1.9	2.9	21.4
		SD	0.1	0.2	0.1	0.1	0.2	0.4	0.0	0.2	0.4	0.2	2.0
Yellow stripey	2	Mean	0.4	0.8	0.5	0.4	3.8	3.6	0.3	1.3	1.6	2.8	21.2
		SD	0.1	0.1	0.1	0.1	1.0	0.0	0.1	0.6	0.0	0.1	0.1

19.9 ± 2.5%, stearic acid (18:0) 8.2 ± 0.9%, and myristic acid (14:0) 2.0 ± 1.1%. There were also small but noteworthy amounts of pentadecanoic acid (15:0), margaric acid (17:0), and arachidic acid (20:0).

MUFA accounted for 17.4 ± 4.3% of total fatty acids. The monoene with the highest concentration was octadecenoic acid (18:1) (11.8 ± 2.6%). The other major monoene was palmitoleic (16:1) (4.3 ± 1.9%). MUFA ranged from 9.6 to 26.1%. This is lower than the variation found by Gibson (14) for fish mostly from Adelaide, latitude 35°S (12.2–68%). The mean MUFA, 22.9 ± 9.7%, for Australian fish calculated by Brown *et al.* (9) is higher than that for Queensland fish in this study ($P < 0.001$); however, for fish caught off the northwest coast of Australia at a latitude of 17°S the mean was 17.0% (13) which indicates that there may be an association between proportion of MUFA and latitude.

TABLE 3
Fatty Acid Analysis of Fish Samples (wt% of total fatty acids)[a]

		Total n-6[b]	Total n-3[c]	n-6/n-3	Total SFA	Total MUFA	Total PUFA
Collared sea bream (*n* = 31)	Mean	17.4	22.5	0.8	31.1	18.1	41.5
	SD	2.8	4.7	0.1	2.5	4.4	6.4
Sweetlip emperor (*n* = 18)	Mean	17.4	21.5	0.8	32.8	18.6	41.0
	SD	3.4	5.7	0.2	3.7	3.8	8.0
Venus tuskfish (*n* = 13)	Mean	17.8	25.2	0.7	31.1	15.8	44.6
	SD	5.4	4.5	0.3	3.8	3.5	6.4
Chickenwire cod (*n* = 11)	Mean	18.3	23.0	0.8	31.6	17.1	42.9
	SD	3.4	5.0	0.2	2.1	3.2	4.4
Yellowtail bream (*n* = 9)	Mean	20.2	24.9	0.8	28.6	13.9	46.4
	SD	3.7	3.7	0.1	2.3	3.8	6.9
Longfin rock cod (*n* = 8)	Mean	17.0	27.0	0.6	30.5	15.8	45.3
	SD	3.7	2.7	0.2	3.6	1.5	3.7
Coral trout (*n* = 7)	Mean	8.8	27.7	0.3	36.8	19.6	37.8
	SD	3.1	4.0	0.1	3.7	4.0	6.6
Red emperor (*n* = 5)	Mean	13.0	28.7	0.5	31.8	17.9	43.0
	SD	1.4	10.3	0.3	4.1	7.6	10.2
Parrot fish (*n* = 4)	Mean	15.6	29.9	0.5	29.5	13.7	46.5
	SD	2.9	4.9	0.1	3.0	3.0	7.2
Hussar (*n* = 3)	Mean	7.8	28.8	0.3	33.5	22.1	38.3
	SD	0.8	2.0	0.0	1.6	2.1	2.6
Yellow stripey (*n* = 2)	Mean	9.4	29.1	0.3	33.8	20.4	39.9
	SD	2.0	0.6	0.1	0.1	4.1	2.9

[a]Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. [b]Includes 18:2. [c]Includes 18:3.

The mean n-6/n-3 ratio for all fish in our study was 0.7 which was higher than the 0.4 calculated for Australian fish by Brown *et al.* (9). This reflects both the higher n-6 PUFA and the lower n-3 PUFA of our samples. The ratios for individual fish varied between 0.2 and 1.4 and the means for species between 0.3 and 0.8.

There was a significant ($P < 0.01$) association between the total lipid content and SFA, MUFA, and PUFA. The proportion of SFA and MUFA rose with increasing lipid content while the concentration of PUFA fell. These relationships are shown in Figure 1. Fogerty *et al.* (16) showed that as the fat content of fish tissue increases beyond 0.8%, triacylglycerols become the predominant lipid. These authors also showed that the phospholipids and free fatty acids are richer in PUFA than the triacylglycerols, thus accounting for the correlations. It is possible that the correlations in the present study may be accounted for in a similar way. These relationships are even stronger when calculated for a single species, collared sea bream, with an appreciable number of samples (*n* = 31). Brown *et al.* (9) showed similar relationships for Australian fish.

No fatty acid profiles have previously been published for at least 9 of the 11 species of Australian fish represented in this study. The previously published mean fatty acid composition of red emperor, *Lutjanus sebae*, from a similar latitude on the other side of the continent (9) was similar to our results. Pearson (10,11) published results for samples of bream but did not give the scientific names for these fish.

The fatty acid profile of fish from this study was similar in many respects to the profile of the average Australian fish as calculated by Brown *et al.* (9). However, although the proportion of PUFA was essentially the same, the relative amount of n-6 PUFA was higher and approximately equal to that reported by Sinclair *et al.* (13) for fish collected from the northwest coast of Australia, which is at similar latitude on the other side of the continent. The proportion of MUFA was lower in this study than that calculated for the average Australian fish but again similar to that of fish from the northwest coast.

This study confirms the findings of others that Australian fish are a good source of both n-3 PUFA and n-6 PUFA and, importantly, shows that there is an association between the total lipid content and the proportions of saturates, monounsaturates, and polyunsaturates.

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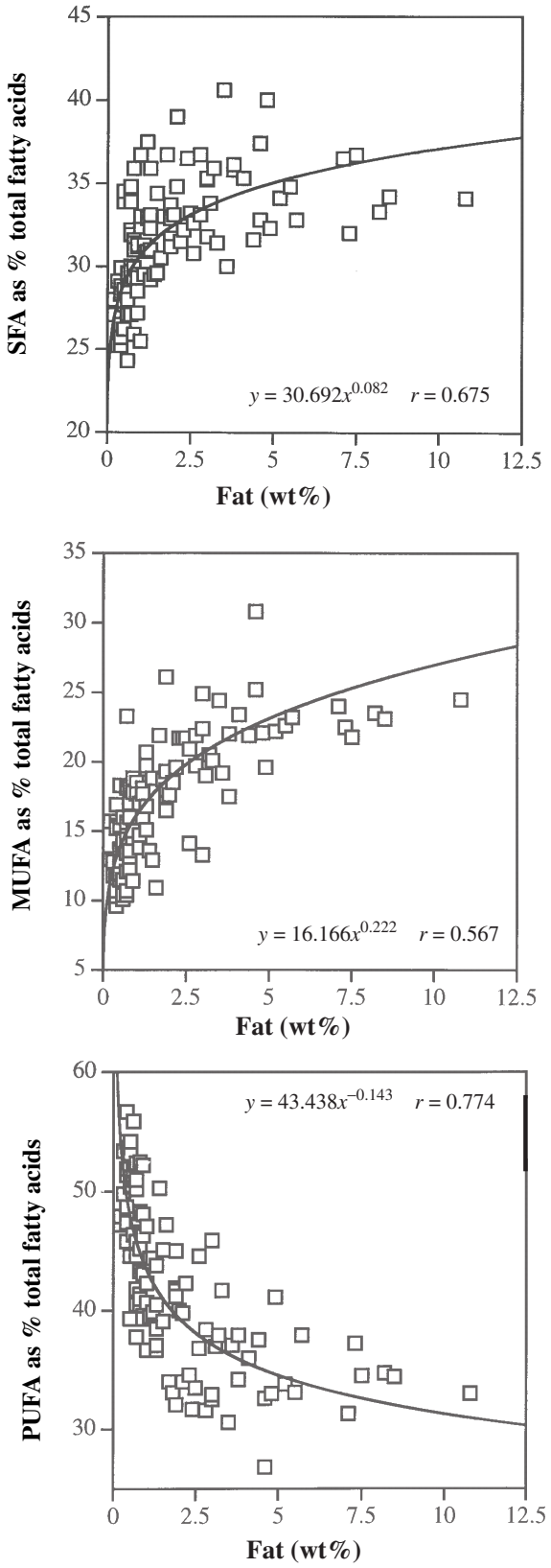

FIG. 1. Correlation between total fat (wt%) and percentage of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) for all fish (n = 111).

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Plasma Kinetic Behavior in Hyperlipidemic Subjects of a Lipidic Microemulsion That Binds to Low Density Lipoprotein Receptors

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ABSTRACT: It was previously reported that a protein-free microemulsion (LDE) with structure roughly resembling that of the lipid portion of low density lipoprotein (LDL) was presumably taken up by LDL receptors when injected into the bloodstream. In contact with plasma, LDE acquires apolipoproteins (apo) including apo E that would be the ligand for receptor binding. Currently, apo were associated to LDE by incubation with high density lipoprotein (HDL). LDE-apo uptake by mononuclear cells showed a saturation kinetics, with an apparent K_m of 13.1 ng protein/mL. LDE-apo is able to displace LDL uptake by mononuclear cells with a K_i of 11.5 ng protein/mL. LDE without apo is, however, unable to displace LDL. The uptake of ^{14}C-HDL is not dislocated by increasing amounts of LDE-apo, indicating that HDL and LDE-apo do not bind to the same receptor sites. In human hyperlipidemias, LDE labeled with ^{14}C-cholesteryl ester behaved kinetically as expected for native LDL. LDE plasma disappearance curve obtained from eight hypercholesterolemic patients was markedly slower than that from 10 control normolipidemic subjects [fractional clearance rate (FCR) = 0.02 ± 0.01 and 0.12 ± 0.04 h^{-1}, respectively; $P < 0.0001$]. On the other hand, in four severely hypertriglyceridemic patients, LDE FCR was not significantly different from the controls (0.07 ± 0.03 h^{-1}). These results suggest that LDE can be a useful device to study lipoprotein metabolism.
Lipids 32, 627–633 (1997).

After degradation of very low density lipoprotein (VLDL) by lipoprotein lipase, only small amounts of triglycerides remain in low density lipoprotein (LDL) particle core. LDL is therefore mostly made up of a core of cholesteryl esters surrounded by a monolayer of phospholipids and unesterified cholesterol. The protein part of LDL consists of a single molecule of apolipoprotein (apo) B100. LDL is removed from the circulation by specific cell receptors, often called B,E receptors, using apo B100 as the ligand (1). Defects in those receptors or in apo B100 may lead to LDL accumulation in the plasma.

Although a great number of LDL plasma kinetic studies in subjects have been performed, the laboratory procedure is time-consuming and impractical because only the autologous lipoprotein can be injected into the subjects. On the other hand, LDL is not a homogeneous fraction and comprises subpopulations of particles with different physical chemical properties and kinetic behavior (2,3). The need of standard preparations to test LDL metabolism was highlighted by Brown and Goldstein (1).

Homogeneous and stable microemulsions without protein with the size and general structure of LDL lipidic portion can be prepared from phospholipids and cholesteryl esters by ultrasonic irradiation of the constituent lipids in aqueous media (4). When apo B100 was associated to the artificial microemulsion, the complex was bound, internalized, and degraded by cultured human fibroblasts in a way similar to native LDL (5). Recently, Maranhão *et al.* (6–8) tested *in vivo* the behavior of the cholesteryl oleate/phosphatidylcholine microemulsion without addition of apo B100 prepared according to the method of Ginsburg *et al.* (4). The physical properties of the microemulsion had been well defined by those authors (4) and others (9). Injected into the circulation of rats, protein-free microemulsion (LDE) showed plasma kinetic behavior, suggesting that it is removed from the plasma by the LDL receptors (6). Although not containing apo B100, LDE picks up several exchangeable apo 1 from the native lipoproteins, and presumably binds to the receptor through apo E, which can be recognized by LDL receptors (10). LDE plasma kinetic behavior showed some similarities with that expected for LDL in rabbits and in humans (7,8). These results raised the possibility that LDE could be used to test *in vivo* LDL receptor function.

In the current study, the evidence that LDE is picked up by the LDL receptors was strengthened by experiments of competition with native lipoproteins for uptake by mononuclear cells. Furthermore, the plasma kinetics of LDE labeled with ^{14}C-cholesteryl ester and injected into subjects with normal

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Abbreviations: AML, acute myeloid leukemia; apo, apolipoprotein; FCR, fractional clearance rate; HDD, high density lipoprotein; LDE, protein-free microemulsion; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

lipid levels as well as hypercholesterolemic or hypertriglyceridemic patients was determined. The results confirm that LDE may be useful to facilitate the study of some aspects of the lipoprotein metabolic pathway.

MATERIALS AND METHODS

Study subjects. Ten normolipidemic healthy subjects (8 males, 2 females, aged 50–67 yr; total cholesterol below 240 mg/dL; LDL up to 130 mg/dL and triglycerides below 250 mg/dL), 8 patients with hypercholesterolemia (7 males, 1 female; aged 53–70 yr; total cholesterol higher than 240 mg/dL; LDL higher than 160 mg/dL and triglycerides below 250 mg/dL), and 4 with severe hypertriglyceridemia (4 males, 32–42 yr; total cholesterol below 240 mg/dL and triglycerides higher than 650 mg/dL) were studied at the outpatient clinic of the Heart Institute of the University of São Paulo Medical School Hospital (São Paulo, Brazil). Controls and cases did not have clinical history of coronary artery disease; diabetes; hyperuricemia; obesity; thyroid, gastrointestinal, kidney or liver disease; or history of excessive alcohol drinking. In the group of hypercholesterolemic patients, those under hypolipidemic drugs had the treatment discontinued for at least 2 mon previously. All the hypertriglyceridemic patients were newly diagnosed, and the LDE clearance experiments were performed before the beginning of the treatment. The experimental protocol was approved by the Ethics Committee of the São Paulo University Medical School, and an informed consent was obtained from each participant.

Cells. Mononuclear cells were isolated from the blood of healthy normolipidemic subjects by centrifugation on a Ficoll-Hypaque gradient (11). Cell viability was >98% as evaluated by the trypan blue exclusion method.

Preparation of the emulsion. Egg phosphatidylcholine was purchased from Lipid Products (Surrey, United Kingdom); triolein, cholesteryl oleate, and cholesterol were from Nu-Chek-Prep (Elysian, MN); and radioactive lipids were acquired from Amersham (United Kingdom). Lipids were >98% pure as determined by thin-layer chromatography. The LDE used in this study was prepared from lipid mixtures composed of 40 mg of phosphatidylcholine, 20 mg of cholesteryl oleate, 1 mg of triolein, and 0.5 mg of unesterified cholesterol. Emulsification of lipids and purification of the emulsions were according to the procedure described by Ginsburg *et al.* (4) as modified by Maranhão *et al.* (8). Whenever possible, all the materials and instruments used in the preparation of LDE were put into an oven at 180°C for 4 h and autoclaved. Lipids were dissolved in chloroform/methanol (2:1) and dispensed into vials. Cholesteryl ^{14}C-oleate was added to the vials. The mixtures were dried under a nitrogen stream followed by overnight vacuum desiccation at 4°C to remove residual solvents. The dried lipids were resuspended in 10 mL of 0.01 M Tris-HCl at pH 8.0. The suspension was sonicated using a Branson Cell Disrupter model 450 (Branson Ultrasonics Co., Danbury, CT) equipped with a flat tip, with a 125-watt output in the "continuous" operating mode, for 180 min under N_2 atmosphere. Temperature was kept above 52°C, the melting point of cholesteryl oleate, as monitored by a thermocouple inserted in the vials during this procedure. The emulsified lipid suspension was then transferred to clean tubes for ultracentrifugation at 195,000 × *g* (30 min) in a TH 641 rotor of a Sorvall OTD-Combi ultracentrifuge (Newtown, CT) at 4°C. The top 10% of the solution, containing particles that float at background density of approximately 1.006 g/mL, was removed by aspiration with a needle. The remaining solution was adjusted to a background density of 1.21 g/mL by adding solid KBr. A second ultracentrifugation step was then performed at 195,000 × *g* (120 min) at 4°C. The top 20–30% of the sample was collected by aspiration, after attaining room temperature, and dialyzed overnight against 10 mM Tris-HCl buffer pH 8 to remove KBr. This LDE fraction (^{14}C-LDE) was sterilized by passage through a 0.2-μ filter. LDE was used in the experiments described below within 4 wk after it was prepared.

Emulsion lipid composition. For the analysis of the emulsion lipid composition, LDE was prepared as described above except that $2.4 \cdot 10^6$ Bq of cholesteryl ^{14}C-oleate, ^{14}C-cholesterol, ^{3}H-triolein, and ^{3}H-phosphatidyl choline were added. The recovery of each lipid was measured after thin-layer chromatography using hexane/ethyl ether/acetic acid (70:30:1, by vol) as moving phase and iodine vapor to develop. Radioactivity of each fraction was measured in scintillation solution (PPO/DM-POPOP/Triton X-100/toluene; 5 g/0.5 g/333 mL/667 mL) using a Packard Spectrometer (Palo Alto, CA). The composition was calculated based on the specific activity of each label.

In vitro *acquisition of apo by LDE.* The emulsion containing apo (LDE-apo) was prepared essentially as described in Reference 6. Briefly, 60 mg of LDE, prepared as described above, was incubated with 30 mg of HDL (protein determined as in Ref. 12) during 2 h at 37°C. After incubation, the density was adjusted to 1.063 g/mL with KBr and the mixture submitted to ultracentrifugation at 195,000 × *g* at 4°C for 4 h. LDE-apo recovered at the top of the tube had acquired 0.7 ng of protein/μg of total lipids. It was labeled with ^{125}I (13) and purified by gel filtration in a PD-10 column (Sephadex G-25m; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) equilibrated and developed 0.15 M NaCl and 1 mM EDTA. With this procedure, 80% of the protein and 20% of the total emulsion lipids were radioactively labeled, and the specific activity obtained was 11,500 cpm/μg protein.

Preparation of high density lipoprotein (HDL) and LDL. HDL and LDL were obtained from the blood of healthy normolipidemic donors by sequential ultracentrifugation (14). After isolation, HDL (1.063–1.21 g/mL) was labeled with ^{14}C-cholesteryl oleate (specific activity obtained = 235 cpm/μg protein) (15), and LDL (1.02–1.063 g/mL) was labeled with ^{125}I (specific activity obtained = 250,000 cpm/μg protein) (13). The purification of the labeled lipoproteins was performed as described above.

Saturation studies. The 10^6 mononuclear cells were incubated with 10–200 ng of protein/mL of ^{125}I-LDE-apo or 10-200 μg/mL of ^{125}I-LDL during 2 h at 37°C, in 1 mL of RPMI-

1640 containing 100 units/mL of penicillin, 5 µg/mL of gentamycin, and 2.5 µg/mL of amphotericin B. After incubation, the cells were washed three times with RPMI 1640 at 4°C, treated with trypsin 0.5 mg/mL for 15 min at 37°C and lysed with 0.2 M NaOH. The incorporated lipoproteins were determined in a gamma counter (COBRA II, Auto-Gamma, Packard, Canberra, Australia).

Competition studies in mononuclear cells. The 10^6 viable cells were incubated with 10 µg/mL of labeled lipoprotein (^{125}I-LDL or ^{14}C-HDL) and increasing concentrations (10–300 µg of lipids/mL) of unlabeled LDE or LDE-apo (7–210 ng of protein/mL) for 2 h at 37°C, in 1 mL of RPMI-1640 containing 100 units/mL of penicillin, 5 µg/mL of gentamycin, and 2.5 µg/mL of amphotericin B. After incubation, the cells were washed and trypsin-treated, and the incorporated ^{125}I-LDL and ^{14}C-HDL were measured as described above.

Clearance studies. The participants were fasting at the commencement of the clearance studies, at approximately 9:00 a.m., but they were allowed two standard meals during the study, at approximately 12:30 p.m. and 7:00 p.m. They were injected intravenously in a bolus with $3.7 \cdot 10^4$ Bq (1 µCi) of radioactive cholesteryl ester, roughly 5–6 mg of emulsion total lipids, in a volume of 500 µL. Plasma samples were collected during 24 h, at 5 min, 1, 4, 12, and 24 h after injection. Aliquots (1.5 mL) of blood plasma were extracted with chloroform/methanol (2:1, vol/vol) (16), and the solvent phase was transferred into counting vials and dried under a nitrogen stream. Radioactivity was measured as described above.

Determination of cholesterol, triglycerides, and apo. Total plasma cholesterol and triglycerides of the subjects were determined after 12 h fasting with the aid of enzymatic kits [CHOD-PAP, Merck (Darmstadt, Germany), and Abbott (Abbott Park, IL), respectively]. HDL-cholesterol was determined by the same method, after precipitation of LDL and VLDL with $MgCl_2$ and phosphotungstic acid. VLDL-cholesterol was calculated assuming the relation: VLDL-cholesterol = (triglycerides/5) (17). LDL-cholesterol was calculated using Friedewald equation where LDL-cholesterol = total cholesterol – (VLDL- cholesterol + HDL-cholesterol) (18). Apo A1 and B were determined by single radial immunodiffusion (19) using Lipo-Partigen (apo A-1) and NOR-Partigen plates (apo B) (Behring, Behringwerke AG, Marburg, Germany).

Calculations. The Michaelis constant K_m for LDL and LDE-apo was calculated by nonlinear regression from the saturation experiments, using the Michaelis-Menten equation and the Grafit program version 3.01 (Robin J. Leatherbarrow-Erithacus Software Ltd., Staines, Middlesex, United Kingdom). The K_i was calculated from the data obtained from the competition in mononuclear cells experiments using the equation:

$$K_i = EC_{50}/(1 + L/K_m) \quad [1]$$

where L = ^{125}I-LDL concentration and LDL K_m = 1.6 µg/mL, as calculated from our saturation experiments. The program GraphPad InPlot version 4.00 (GraphPad Software Inc., San Diego, CA) was used for these calculations.

The experimental curves of the plasma decay of the cholesteryl ^{14}C-oleate radioactivity showed a biexponential aspect. The decay curve was analyzed by its decomposition in exponential components, initially by curve peeling. The profile of the experimental curves plotted in semilogarithmic graphic suggested that the last points of the curves fitted to a straight line. The intersection of this straight line with the y-axis defined the a_2 parameter, and its slope is defined as b_2. Each experimental point was then subtracted from the corresponding value on the straight line. These new data points were fitted to a second straight line, defining the slope b_1 and the intercept a_1. To obtain accurate values for these parameters, they were used as seeds for the subsequent process of fitting using the nonlinear least squares method applied to the function:

$$y = (a_1 \cdot e^{-b_1 x}) \cdot (a_2 \cdot e^{-b_2 x}) \quad [2]$$

The intercept parameters were normalized to obtain $a_1 + a_2 = 1$. The fractional clearance rate (FCR) is calculated according to the method described by Matthews (20), using the equation:

$$FCR = \left(\frac{a_1}{b_1} + \frac{a_2}{b_2}\right)^{-1} \quad [3]$$

Differences in LDE FCR, triglycerides, and total cholesterol and fractions between the groups were assessed using the nonparametric Mann-Whitney test (21). Two-tailed P values below 0.05 were considered significant.

RESULTS

Emulsion lipid composition. Prepared as described, the percentage molar composition of the emulsion is 48.0% cholesteryl esters, 47.8% phospholipids, 2.3% triglycerides, and 1.9% unesterified cholesterol.

Uptake of LDE-apo and LDL by mononuclear cells. Figure 1 shows the uptake of ^{125}I-LDE-apo (1A) and ^{125}I-LDL (1B) by mononuclear cells. A saturation kinetics was observed with a K_m of 13.1 ng/mL for LDE-apo and a K_m of 1.6 µg/mL for LDL.

In vitro *competition studies.* Figure 2B shows that the addition of increasing amounts of LDE does not inhibit the uptake of ^{125}I-LDL by mononuclear cells. However, when LDE acquires apo by pre-incubation with HDL, ^{125}I-LDL uptake is then progressively inhibited by LDE-apo (Fig. 2A). The K_i of LDE-apo is 11.5 ng/mL. This indicates that LDE-apo, but not LDE, is capable of competing with the native lipoprotein for binding to LDL receptors.

As it is shown in Figure 2C, the HDL cholesteryl ester oleate uptake by mononuclear cells is not inhibited by LDE-apo. Conversely, HDL did not inhibit LDE-apo uptake (Fig. 2D). These results indicate that the B/E receptor is the unique site of uptake of LDE-apo in this cell type.

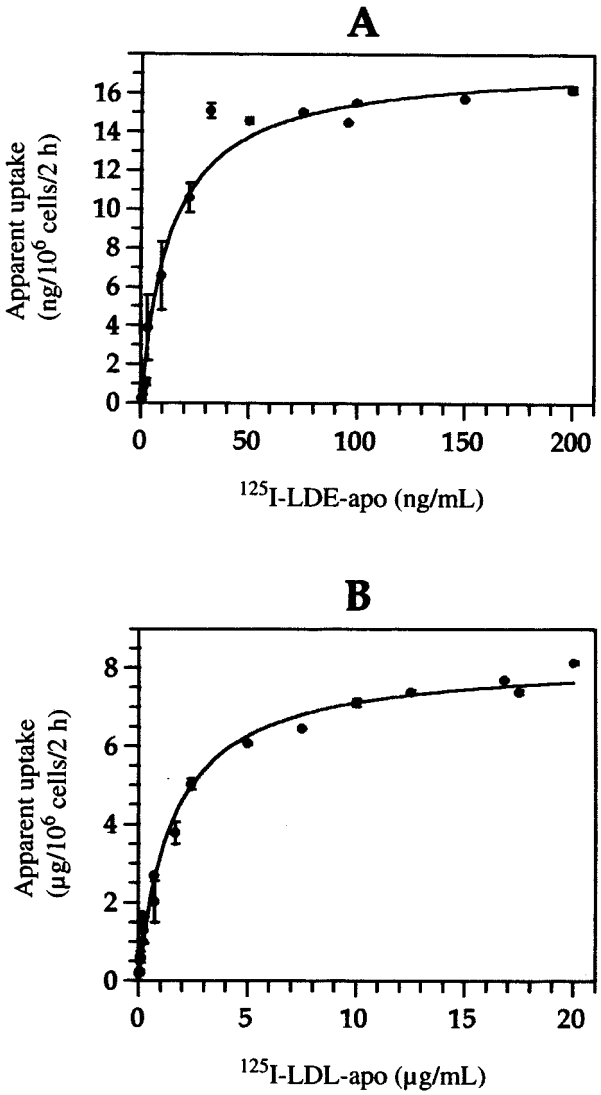

FIG. 1. Uptake of ^{125}I-LDE-apo and iodine-labeled low density lipoprotein (^{125}I-LDL) by mononuclear cells. 10^6 mononuclear cells were incubated with 10–200 ng of protein/mL of iodine-labeled protein-free microemulsion (^{125}I-LDE)-apo (A) or 10–200 µg/mL of ^{125}I-LDL (B) during 2 h at 37°C, in 1 mL of RPMI-1640. After incubation, the cells were washed, treated with trypsin, lysed, and the incorporated lipoproteins were determined in a gamma counter. Each point represents mean ± SEM of three experiments. The K_m determined were 13.1 ng/mL and 1.6 µg/mL for LDE-apo and LDL, respectively.

Plasma lipids and LDE kinetics. Table 1 shows the plasma lipids for the three groups studied (mean ± SEM). Figure 3A shows that the decay curve of ^{14}C-cholesteryl ester LDE obtained in patients with moderate hypercholesterolemia is pronouncedly slowed when compared to controls. In the patients with severe hypertriglyceridemia, the ^{14}C-cholesteryl ester LDE curve tended to approach that of the controls.

FCR was significantly different in the hypercholesterolemic patients compared with controls ($P < 0.001$) or the hypertriglyceridemic subjects (0.02 ± 0.01; 0.12 ± 0.01; and 0.07 ± 0.03 h^{-1}, respectively; Fig. 3B).

DISCUSSION

Several experimental observations have conveyed the concept that LDE is picked up by LDL receptors (6–8). When incubated with HDL, several exchangeable apo shift from the lipoprotein to the artificial emulsion (6) including apo E that could enable LDE binding to the LDL receptor (10). The emulsion clearance was accelerated in rats pretreated with 17α-ethinylestradiol which enhances the expression of these receptors (22). ^{99m}Technetium-labeled LDE biodistribution images showed a marked resemblance with those obtained with LDL in the rabbit as well as in humans. Liver is the predominant uptake organ. Furthermore, LDE had increased removal from plasma in patients with acute myeloid leukemia (AML) (7,8) as occurs with native LDL because receptors are upregulated in AML cells (23,24). Conversely, destruction of the AML cells by chemotherapy resulted in decreased LDE clearance, which then approaches normal values (8). LDE clearance was also increased in postmenopausal women after estrogen replacement therapy (25), as expected, since estrogen increases LDL receptors (26).

In the current study, the set of experiments performed with mononuclear cells provided direct evidence that LDE is indeed specifically taken up by the LDL receptors. First, it was shown that LDE-apo was taken up by the cells through a saturable mechanism. Second, the results clearly indicate that LDE-apo strongly competes with native LDL for cell uptake. The incorporation of apo was proven necessary to endow the emulsion with capacity to compete with LDL for the cell receptors. The experiment showing that the emulsion does not compete with HDL for cell uptake sites offers an additional assurance for the specificity of the LDE binding to the LDL receptors. This is important because it could be speculated that LDE could also interact with the HDL uptake mechanisms (27).

In the current investigation, the possibility of using the microemulsion as a tool to explore lipid metabolism was confirmed by determining LDE kinetics in primary hypercholesterolemia. In this condition, the receptor activity is decreased, resulting in reduction of LDL clearance, as documented in a number of studies by the injection of radioactive iodine-labeled LDL (28). Therefore, the reduction of LDE FCR found in our study is the effect expected for native LDL.

In hypertriglyceridemia, the generation of VLDL catabolic products is decreased because lipoprotein lipase function is defective. Poorly catabolized VLDL is not efficiently removed by the LDL receptor mechanisms, which are intrinsically normal. Therefore, the fact that LDE decay curve of three extremely hypertriglyceridemic patients was not different from that of the controls would also be expected for native LDL, as previously described (28).

LDE rates of removal from plasma appear much greater than those of native LDL. As showed in Figure 3A, 2 h after injection roughly 50% of LDE radioactivity had already been eliminated from the plasma of the controls. Lipoproteins containing multiple copies of apo E have higher affinity than apo

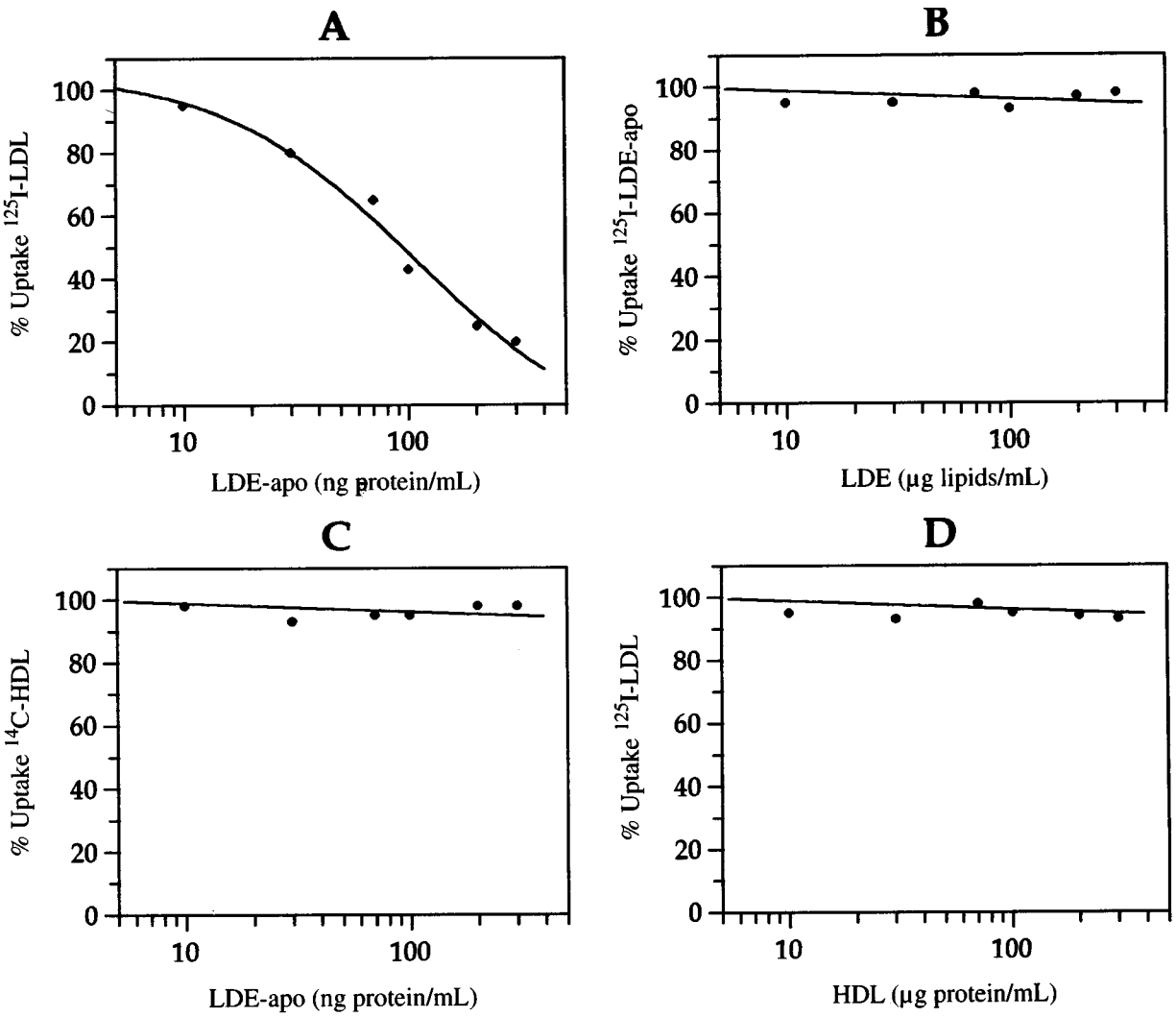

FIG. 2. Competition studies. Mononuclear cells were incubated with 10 µg/mL of ^{125}I-LDL (A and B), ^{14}C high density lipoprotein (HDL) (C), or ^{125}I-LDE apoprotein (apo) (D) and increasing amounts of LDE-apo (A and C), LDE (B) or HDL (D). ^{125}I-LDL uptake was inhibited only by LDE-apo (A), with a K_i of 11.5 ng protein/mL. HDL and LDE-apo did not compete for the same uptake sites (C and D). See Figure 1 for other abbreviations.

B for binding to the LDL receptor (10). Indeed, when apo B100 was associated to LDE and the complex injected into rats, the emulsion clearance was markedly decreased (29). We previously showed in rats that the presence of free cholesterol in the composition of microemulsions resembling LDL accelerates its removal from the plasma (30). This is an example of the operational advantages that an artificial emulsion system may offer. By manipulating the composition of the emulsion, it is possible to optimize experimental conditions or systematically observe the effect of one isolated component on the emulsion behavior. Acceleration of plasma clearance may be advantageous since the duration of the test can be shortened from some days, as with native LDL, to 24 or 12 h. In addition, artificial emulsion systems allow easy labeling with isotopes or other markers for the performance of metabolic studies.

TABLE 1
Lipid Profile and FCR of the Three Studied Groups[a]

Group (n)	Cholesterol (mg/dL)			TG	Apolipoprotein (g/L)[b]		
	Total	LDL	HDL	(mg/dL)	A1	B	FCR (h^{-1})
CT (10)	193 ± 10	118 ± 9	44 ± 4	128 ± 16	1.2 ± 0.2	1.2 ± 0.3	0.12 ± 0.01
Hch (8)	272 ± 11[d]	190 ± 10[d]	42 ± 2	166 ± 20	1.5 ± 0.3	1.8 ± 0.3[c]	0.02 ± 0.01[d]
Htg (4)	202 ± 16	n.d.	36 ± 1	2413 ± 1110[b]	0.9 ± 0.2[c]	1.0 ± 0.6	0.07 ± 0.03

[a]The values represent mean ± SEM; n.d., not determined; CT, control; Hch, hypercholesterolemic; Htg, hypertriglyceridemic; FCR, fractional clearance rate; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglycerides. [b]$P < 0.01$. [c]$P < 0.05$. [d]$P < 0.001$.

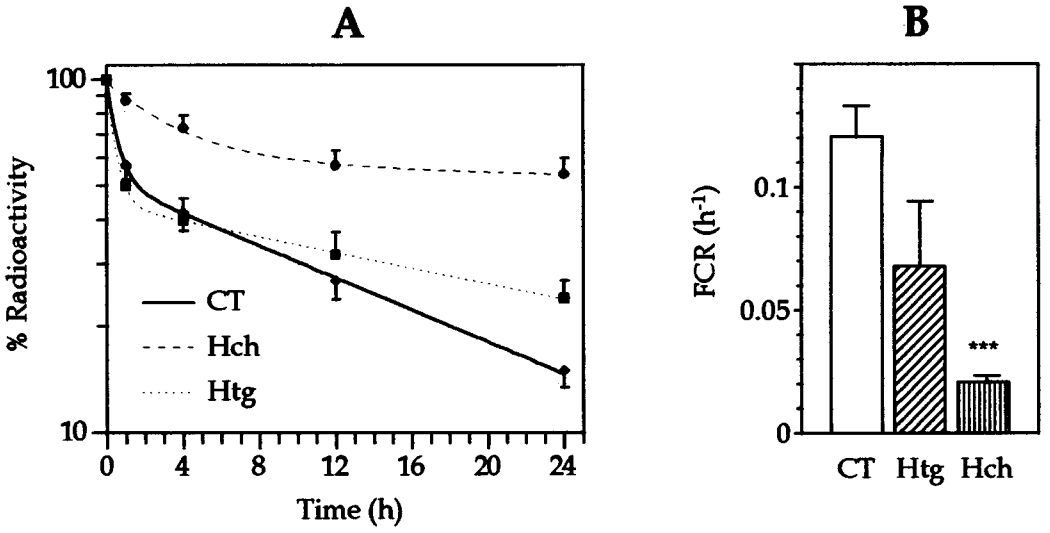

FIG 3. (A) Plasma kinetics of ^{14}C-LDE in normal subjects, hypertriglyceridemic (. . . . .), and hypercholesterolemic (-------) patients. Points represent mean ± SEM. The fractional clearance rate (FCR) (mean ± SEM) is shown in (B); CT = controls; Htg = hypertriglyceridemic; Hch = hypercholesterolemic patients. *** $P < 0.0001$.

There are three common isoforms of human apo E: apo E2, E3 and E4 (31). Roughly 70% of the Caucasian population carries the E3 variant, whereas the E4 isoform is the rarest. Apo E3 and E4 have similar affinity to the LDL receptor, but apo E2 has only 10% of the affinity of the other two. It is thus presumable that in the minority of persons possessing apo E2, LDE clearance should be smaller, especially in the homozygous form.

LDL accumulation and hypercholesterolemia can also result from genetically inherited defects of apo B100 affecting the binding domains of the apo (32). In those cases, LDE removal from plasma would not be expected to be altered, since the emulsion is recognized by the receptor for apo E. Therefore, the emulsion could eventually be used to screen for the existence of functional abnormalities of apo B or distinguish them from defects related to LDL receptor function.

In the current study, LDE was shown capable of specifically testing LDL receptor function in hyperlipidemic patients. As monitored by visual inspection and by repeated determination of the ratio of the isotopically labeled components, as well as by serial determination of its kinetics in rats, LDE is stable for at least 1 mon when kept at 4°C. Addition of antioxidants is likely to extend the long-run stability. Therefore, with a single LDE preparation, it is possible to study a great number of individuals over a long period of time. LDE may then be suitable for studies aiming to disclose disturbances of lipoprotein metabolism and lipoprotein receptor, pathophysiological processes, drug treatments, or dietary regimens.

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The Effect of Short-Term Diets Rich in Fish, Red Meat, or White Meat on Thromboxane and Prostacyclin Synthesis in Humans

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ABSTRACT: Foods which increase tissue arachidonic acid levels have been proposed to increase thrombosis tendency, presumably through increased platelet aggregation. This study examined the effect of doubling the dietary arachidonic acid (20:4n-6) using meat- or fish-based diets on the systemic production of prostacyclin (PGI_2) and thromboxane (TXA_2) in 29 healthy, nonsmoking adults. There were three, 3-wk low-fat dietary periods (<15% energy as fat) in which subjects consumed a vegetarian diet for 1 wk followed by 2 wk on diets containing meat or fish as sources of 20:4n-6. Between each diet period, there was a 3-wk washout period, during which subjects returned to their normal diets. The level of 20:4n-6 consumed during the last 2 wk of each study was approximately double the usual intake (mean 140 mg/d), while the mean eicosapentaenoic acid (20:5n-3) content of the diets varied from 1 mg/d on the white meat diet to 70 mg/d on the red meat diet and to 847 mg/d on the fish diet. The serum phospholipid (PL) 20:4n-6/20:5n-3 ratios were 11:1 on the vegetarian diet, 15:1 on the white meat diet, 8:1 on the red meat diet, and 2:1 on the fish diet ($P < 0.001$). Neither white nor red meat diets affected platelet 20:4n-6 levels, platelet aggregation, *ex vivo* platelet TXB_2 production, or the systemic PGI_2 or TXA_2 production as measured by gas chromatography–mass spectrometry analysis of the excretion levels of the principal urinary metabolites 2,3-dinor-6-keto-$PGF_{1\alpha}$ (PGI_2-M) and 11-dehydro-TXB_2 (TXA_2-M), respectively. The fish diet decreased the 20:4n-6/20:5n-3 ratio in platelet PL from the baseline level of 45:1 to 13:1 ($P < 0.001$), had no effects on platelet aggregation, but significantly decreased platelet TXB_2 production (collagen-stimulated) and TXA_2-M production, while PGI_2-M levels were unaltered. These results indicate that short-term diets which double the usual 20:4n-6 intake using white meat (175–330 g/d) or red meat (275–530 g/d) are not associated with an increased TXA_2 production, but this does not rule out the adverse effects of 20:4n-6 at higher levels in the diet, or for more prolonged periods. Short-term diets containing fish (100–200 g/d with 90–210 mg/d 20:4n-6 and approximately 650–1000 mg/d 20:5n-3) led to significant increases in platelet 20:5n-3 levels and a decrease in the *ex vivo* and systemic TXA_2 production.

Lipids 32, 635–644 (1997).

Eicosanoids derived from arachidonic acid (20:4n-6) are thought to play a role in the pathogenesis of coronary artery disease. The two most powerful vasoactive eicosanoids issuing from the cyclooxygenase pathway are the platelet-derived thromboxane (TXA_2) (1) and prostacyclin (PGI_2), derived from arterial endothelial cells (2). Thromboxane is both a potent pulmonary vasoconstrictor and a procoagulant, whereas PGI_2 is a major endogenous vasodilator and an inhibitor of platelet aggregation (3,4). It is believed that an imbalance between the release of these two mediators could be involved in the pathogenesis of thrombogenesis and atherosclerosis (5).

It has been proposed that the low incidence of myocardial infarction and ischemic heart disease in Greenland Eskimos is due to the high content of long-chain (LC) n-3 polyunsaturated fatty acids (PUFA) in their diet (6,7). The beneficial effects may be due to a decrease in TXA_2 derived from 20:4n-6 and an increase in the 3-series prostanoids which have an attenuated spectrum of biological activity (8). The thromboxane/prostacyclin ratio has subsequently been shown to be reduced in Greenland Eskimos compared with Danes (9).

Since dietary LC n-3 PUFA modify eicosanoid formation from the more dominant precursor fatty acid, 20:4n-6, it has been argued that the ingestion of foods which raise tissue levels of 20:4n-6 will lead to an increased thrombotic potential (10). This view has been based to a large extent on the detrimental findings of two studies, the first of which involved rabbits being injected intravenously with 20:4n-6, resulting in death within 3 min (11). The second was a human study in which four subjects were fed 6 g/d of ethyl arachidonate for a period of 2–3 wk. The threshold concentration of ADP re-

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Abbreviations: 20:4n-6, Arachidonic acid; DHA, docosahexaenoic acid (22:6n-3); LA, linoleic acid; LC, long-chain; PAP, platelet adjusted plasma; PEG, polyethylene glycol; PGI_2, prostacyclin; PGI_2-M, 2,3-dinor-6-keto-$PGF_{1\alpha}$; PL, phospholipid; PPP, platelet-poor plasma; PRP, platelet-rich plasma; P/S, polyunsaturated/saturated; PUFA, polyunsaturated fatty acid; TXA_2-M, 11-dehydro-TXB_2; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2.

quired to induce secondary irreversible aggregation of platelet-rich plasma (PRP) dropped significantly in two of the four subjects, indicating increased platelet reactivity (12).

The Western diet is rich in 18:2n-6, containing 10–30 g/d, compared with 1–3 g/d 18:3n-3 (13) and about 50–70 mg/d 20:5n-3 and 100 mg/d docosahexaenoic acid (DHA) (14). The 20:4n-6 intake in the Western diet has been postulated to be between 100–1000 mg/d (15,16). We have reported the 20:4n-6 intake in the Australian diet to be approximately 130 mg/day for adult males and 96 mg/day for adult females (14), based on average national food intake figures. Thus, the relatively high level of n-6 PUFA in the diet, coupled with the body's natural tendency to conserve tissue arachidonic acid, leads to 20:4n-6 being the most abundant 20-carbon PUFA available in tissue membrane phospholipids (PL) for eicosanoid synthesis.

The relative importance of dietary 20:4n-6, compared with dietary linoleic acid, in contributing to tissue 20:4n-6 levels is not fully understood; however, we have shown that short-term diets (1 to 3 wk) rich in foods containing up to 500 mg/day of 20:4n-6 (red meat, tropical seafood, eggs, liver) are associated with significant increases in the plasma levels of 20:4n-6 (17–20). Furthermore, we have recently reported data from a pilot study indicating that such diets are associated with alterations in thromboxane and prostacyclin production (21).

The objectives of this study were to investigate the effect of three diets containing 20:4n-6 (from foods) on serum and platelet PUFA levels, *in vitro* platelet thromboxane production, and *in vivo* whole body production of PGI_2 and TXA_2. The three diets with elevated but constant levels of 20:4n-6 contained low, medium, and high levels of LC n-3 PUFA. Rather than using isolated oils in pharmacological doses, a more realistic dietary approach was used: The three diets all contained the same level of 20:4n-6 (mean of 140 mg/day) which was approximately double the usual consumption level of 20:4n-6 for these particular subjects, whose normal diets were relatively low in meat and eggs, the main source of dietary 20:4n-6. The first diet was rich in white meat which provided little LC n-3 PUFA, the second diet was rich in red meat which provided a similar level of n-3 PUFA to the usual diet, and the third diet was rich in fish which provided approximately 3 g/day of n-3 PUFA.

MATERIALS AND METHODS

Subjects. A total of 29 free-living subjects took part in the three phases of the intervention study (14 males, 15 females), with an age range of 22–52 yr. Subjects were screened for any existing medical condition and requested not to consume garlic, which is known to affect lipid and cholesterol levels and platelet activity (22), or use any form of medication during the study, particularly corticosteroids and nonsteroidal anti-inflammatory drugs such as aspirin, which could interfere with cyclooxygenase activity (23), thus altering platelet function. Subjects were also requested to abstain from extreme exercise and the consumption of vitamin and mineral supplements (particularly vitamin E) during the study and 2 wk prior to the start.

The study consisted of three 3-wk low-fat (<15% energy as fat) dietary intervention phases with a 3-wk washout period between each, at which time subjects returned to their own "baseline or normal" diet. Individual phases consisted of 1 wk of precooked vegetarian food, followed by 2 wk of meat consumption (either white meat, red meat, or fish). Vegetarian meals were precooked for subjects due to their lack of familiarity with vegetarian cooking. Meat was provided raw and cooked by subjects to their own tastes (no added fats) and consumed with vegetables if desired. The white meat consisted of fat-trimmed turkey fillets (<7.2% fat) and turkey roll (1% fat); the red meat consisted of fat-trimmed beef (<2.8% fat) and lamb (<4.2% fat), and the fish was Atlantic salmon (<10% fat) (14). The average intakes were 231 ± 52 g/day of white meat, 351 ± 104 g/day red meat, and 133 ± 32 g/day of fish (Fig. 1). The short duration of each study (2 wk) was largely due to the difficulty that subjects found in maintaining the consumption levels of meat continuously over an extended period of time.

The fish diet was the last carried out, as tissue DHA levels are known to take up to 3 wk to return to pre-dietary intervention levels, whereas other LC PUFA return to normal levels more quickly (24). No other fat sources were allowed and energy percentage as fat was made up to 15% for each individual with olive oil (77% oleic acid, Moro Spanish Olive oil; Conga Foods, Melbourne, Australia) or Brio™ margarine (Brio Foods, Pireas, Greece) containing 61% oleic acid and 13% *trans*-18:1.

The daily energy intake of each subject was determined from pre-intervention study weighed food records, and the dietary 20:4n-6 intake was designated to be proportional to the total energy intake of each subject. The white meat, red meat, and fish stages of the study were thus controlled for 20:4n-6 intake, but with varying levels of LC n-3 PUFA. No LC PUFA were consumed during the vegetarian periods, so that all subjects entered each dietary period from the same background PUFA intake, giving a valuable reference point for the purpose of examining comparative changes on each diet. Also no LC PUFA were available from any dietary source other than the meat or fish during the study periods. Dietary composition was calculated using SODA (System for On-line Dietary Analysis, version 5.0; Computer Models, Cottesloe, WA., Australia) based on Australian food composition data.

Blood collection. Subjects reported to the clinical rooms of the University in the morning, following a 12-h overnight fast on three occasions during each phase of the study. These were at the beginning of each phase, at the end of week one (vegetarian diet), and again at the end of week three, after 2 wk of meat or fish consumption. Weight was recorded to the nearest 0.1 kg. At these times they received dietary counseling and their records of food consumed (by weight) were collected, checked and any relevant changes in diet for the following week explained to them. Subjects were requested to

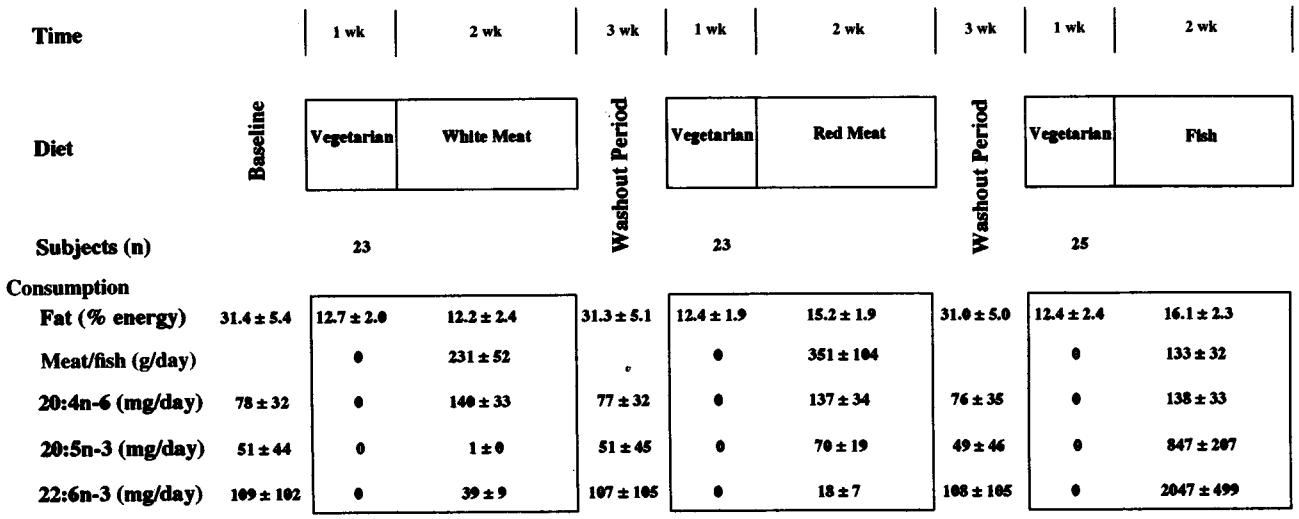

FIG. 1. Experimental design. Three dietary regimes were examined, each consisting of a 1-wk vegetarian diet followed by 2 wk of white meat, red meat, or fish consumption. Each phase was separated by a 3-wk washout period to allow serum phospholipid fatty acid levels to return to baseline. The percentage energy derived from fat in each stage of the study is indicated along with a summary of the amount of meat or fish consumed, with the relevant content of arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3), and docosahexaenoic acid (22:6n-3). Values are mean ± SD.

report any deviations from the dietary protocol and any signs of illness.

Blood was collected following 15 min of quiet relaxation, with minimum stasis. Approximately 34 mL of blood was collected sequentially into a 10-mL plain vacuette (Griener Interpath, Melbourne, Australia), followed by two 5-mL CTAD vacuettes (citric acid, theophylline, adenosine, dipyridamole tubes; Becton Dickinson, Oxford, United Kingdom) pre-warmed to 37°C, and finally three 5-mL sodium citrate vacuettes (Griener Interpath). The serum was removed and frozen at −20°C for later determination of serum fatty acid composition. The blood collected in the three citrate tubes was used for full blood examination and platelet aggregation studies.

Serum PL fatty acid analysis. Lipids were extracted from the serum, and the total PL were separated by thin-layer chromatography and fatty acid methyl esters (FAME) were prepared by saponification in 0.68 M KOH in methanol, followed by methyl esterification with 20% BF_3 in methanol (25). The amounts were quantified using diheptadecanoyl phosphatidylcholine as an internal standard. The FAME were separated by capillary gas chromatography using a 50 m × 0.32 mm i.d. BPX70, WCOT fused-silica capillary column (SGE, Melbourne, Australia) as described previously (26).

Platelet PL fatty acid analysis. Platelets were separated from 10 mL of blood collected in CTAD vacutainers (27) as described by Naughton *et al.* (28), and extracted in 10 mL of 1:1 chloroform/methanol. All platelet separation steps were carried out in polypropylene or silanized glass tubes, and PL fatty acid analysis proceeded as for serum PL fatty acids.

Plasma preparation for platelet aggregation. Platelet-poor plasma (PPP) and PRP were prepared as described previously by Steel *et al.* (29). Platelet aggregation studies required plasma with equal quantities of platelets for each subject, hence requiring concentration adjustment. The platelet-adjusted plasma (PAP) used was obtained by individual dilution of each subject's PRP with their own PPP until a platelet concentration of 220×10^9 platelets/L was achieved. The PAP was then stored at 37°C until the aggregation test was performed, within 2 h of the blood collection.

Platelet aggregation and platelet thromboxane production. Platelet aggregation was determined in PAP using a dual channel, Payton Aggregation Module 300 BD-5 (Payton Scientific Pty. Ltd., Victor Harbour, South Australia). The method used was a turbidimetric method, and aggregation was measured using two different agonists, collagen (Hormon Chemie GMBH, Munich, Germany) and thrombin (Thrombostat, Parke Davies Pty. Ltd., Caringbah, NSW, Australia), both of which initiate a TXA_2 release.

Following platelet aggregation, the supernatant of the aggregated PAP was removed and used for thromboxane determination as outlined by Butcher *et al.* (30). TXB_2 was determined in duplicate in each sample using a radioimmunoassay technique (Scintillation Proximity Assay, SPA, Amersham, Melbourne, Australia) and measured on a liquid scintillation counter. All samples were analyzed as one batch and a pooled serum sample was analyzed five times to determine the intraassay coefficient of variation (mean ± SD, 720.5 ± 8.0 cpm, coefficient of variation 1.1%).

Urine collection. Twenty-four-hour urine samples were collected by subjects, to which was added Indomethacin (Sigma Chemical Co., St. Louis, MO) (50 mg/50 mL urine). Aliquots of 50 mL were stored at −80°C until required for extraction and derivatization.

Prostacyclin measurement. The mean daily excretion of PGI_2-M (2,3-dinor-6-keto-$PGF_{1\alpha}$) derived from 20:4n-6 and

PGI_3-M (Δ^{17}-2,3-dinor-6-keto-$PGF_{1\alpha}$) derived from 20:5n-3 was determined as the methyloxime, pentafluorobenzyl ester, trimethylsilyl ether derivative in 5-mL aliquots of urine spiked with 10 ng of tetradeuterated [19,19,20,20-$^2H_4$] 2,3-dinor-6-keto-$PGF_{1\alpha}$ as an internal standard as described previously (31). The samples were separated using a 12-m, 0.22-mm i.d. BP-1 fused-silica capillary column (SGE, Melbourne, Australia), which interfaced directly with a Hewlett-Packard 5988A single quadrupole mass spectrometer (Palo Alto, CA) as outlined previously (32). The interassay coefficient of variation was 4.2%.

Thromboxane measurement. Quantitative analysis of 11-dehydro-TXB_2 in urine was used for the assessment of *in vivo* thromboxane production (33). A 2-mL aliquot of urine was spiked with 1 ng of tetradeuterated [19,19,20,20-$^2H_4$]-11-dehydro-TXB_2 as an internal standard. Gas chromatography was performed on a Varian 3400 (Palo Alto, CA) operated in splitless mode with a fused-silica capillary column, DB5-MS, 30 m, 0.25 mm i.d., 0.25 mm coating (J&W Scientific, Folsom, CA). The gas chromatograph was interfaced to a Finnigan MAT TSQ 70 triple-stage mass spectrometer (San Jose, CA) operated in NICI mode with methane used as ionization gas. Selected ion monitoring was used to monitor the molecular ion of the pentafluorobenzyl ester trimethylsilyl ether forms of the endogenous metabolite 11-dehydro-TXB_2 and the tetradeuterated [19,19,20,20-$^2H_4$]-11-dehydro-TXB_2 internal standard at *m/z* 511 and *m/z* 515, respectively.

Statistical analysis. We analyzed the results using analysis of variance on data blocks between baseline, vegetarian, and diet period during each study. As sample sizes were approximately equal, the Student's Newman Keul multiple comparison test was applied. All statistical analyses were carried out on a Sun Sparc Station, running Unix, using SAS (Statistical Analysis System, Cary, NC).

RESULTS

One male and one female subject failed to complete the white meat phase of the study, and their results were not recorded; another male only completed the white meat study. One extra female was recruited for the red meat phase, and all subjects completed this phase of the study. One male and two females did not continue with the third phase of the study. Two extra males and three females were recruited to take part in the fish phase of the study. Overall, nine females and 10 males completed all three phases of the study. All subjects remained within a 2-kg weight range during their participation. No statistically significant change occurred in group weight or body mass index in any phase of the study (Table 1). There were no significant differences in baseline levels of PL fatty acids or *in vivo* production of thromboxane or PGI_2 at the start of each study, indicating a return to normal levels during the washout periods.

Fat consumption as a percentage of energy intake on baseline diets was 31% (all figures quoted are for combined male and female results, unless otherwise stated) (Table 2). After 1 wk of vegetarian diet during each of the three diet phases, the fat levels were 12–13% of energy. Following 2 wk on each of the diets, the dietary fat levels were 12, 15, and 16% of energy intake, respectively. The marked fall in the proportion of energy derived from fat on all diets was compensated for by increased carbohydrate consumption.

There was a substantial decrease, relative to baseline, in saturated fat intake during each phase of the study (Table 2). The decrease in monounsaturated fatty acid, relative to baseline during each phase, was more modest owing to the use of olive oil in making up each individual diet to 15% energy as fat. The baseline polyunsaturated/saturated (P/S) ratio in each phase was 0.4, which increased to 0.9 during each of the three vegetarian periods. During the white meat phase, the P/S ratio remained at 0.9, but fell to 0.5 during the red meat phase. The fish was relatively rich in PUFA, and during this phase of the study the dietary P/S ratio increased to 1.3. Cholesterol intake was extremely low on the vegetarian diets relative to baseline ($P < 0.001$). During the white meat and fish phases of the study, cholesterol intakes rose relative to the vegetarian period ($P < 0.001$), but were still well below baseline intakes ($P < 0.001$). During the red meat phase of the study, cholesterol intake returned to baseline levels.

The quantity of meat or fish consumed by each subject and

TABLE 1
Subject Numbers, Age, Weight, and Body Mass Index (BMI) for the White Meat, Red Meat, and Fish Dietary Intervention Studies[a]

	Phase of Study											
	White meat				Red meat				Fish			
	n	Age (yr)	Weight (kg)	BMI	n	Age (yr)	Weight (kg)	BMI	n	Age (yr)	Weight (kg)	BMI
Males	12	34.9 ± 10.0	79.5 ± 5.2[c]	24.5 ± 1.3[b]	11	35.8 ± 9.9	80.2 ± 4.3[c]	24.7 ± 0.7[b]	12	34.8 ± 9.5	77.9 ± 4.4[c]	24.5 ± 0.8[b]
Females	11	34.7 ± 8.5	63.3 ± 7.6	22.8 ± 2.4	12	35.0 ± 8.1	63.4 ± 7.3	23.0 ± 2.3	13	35.8 ± 7.4	61.8 ± 8.5	22.5 ± 2.4
All subjects	23	34.8 ± 9.1	71.7 ± 10.4	23.7 ± 2.1	23	36.8 ± 13.2	71.4 ± 10.4	23.8 ± 1.9	25	35.4 ± 8.3	69.5 ± 10.6	23.5 ± 2.1

[a]Values are mean ± SD.

[b,c]Significant difference between males and females ([b]$P < 0.01$, [c]$P < 0.001$).

TABLE 2
Changes in Dietary Composition During the White Meat, Red Meat and Fish Dietary Intervention Studies

	Stage	Energy intake (kj/day)	Carbohy.	Protein	Alcohol	Fat	Sat.	Mono.	Poly.	Cholesterol (mg/day)	Fiber (g/day)
			(percentage of daily total energy intake)								
White meat (n = 23)	Baseline	9,637 ± 2,439	49.4 ± 5.2	16.7 ± 2.1	2.6 ± 3.8	31.4 ± 5.4	14.5 ± 3.0	11.8 ± 3.2	5.2 ± 1.7	235 ± 78	27.5 ± 9.8
	Week 1	9,918 ± 2,137	72.8 ± 5.6^{c}	11.8 ± 2.4^{c}	2.6 ± 3.9	12.7 ± 2.0^{c}	2.9 ± 0.5^{c}	7.5 ± 1.3^{c}	2.5 ± 0.5^{c}	15 ± 11^{c}	43.6 ± 11.1^{c}
	Week 3	9,673 ± 1,862	65.0 ± 6.4^{c,f}	20.7 ± 3.1^{c,f}	2.2 ± 2.7	12.2 ± 2.4^{c}	2.8 ± 0.5^{c}	6.9 ± 1.5^{c}	2.4 ± 0.5^{c}	144 ± 33^{c,f}	31.4 ± 8.4^{c,f}
Red meat (n = 23)	Baseline	9,451 ± 2,483	49.0 ± 6.0	16.8 ± 2.2	3.0 ± 4.0	31.3 ± 5.1	14.4 ± 3.2	11.8 ± 2.8	5.1 ± 1.7	227 ± 68	26.5 ± 10.4
	Week 1	9,611 ± 2,372	72.9 ± 4.2^{c}	12.0 ± 1.7^{c}	2.8 ± 3.9	12.4 ± 1.9^{c}	2.6 ± 0.4^{c}	7.6 ± 1.4^{c}	2.2 ± 0.4^{c}	14 ± 10^{c}	37.7 ± 11.4^{c}
	Week 3	9,404 ± 2,182	59.4 ± 4.3^{c,f}	22.7 ± 2.2^{c,f}	2.7 ± 3.0	15.2 ± 1.9^{c,f}	4.6 ± 0.5^{c,f}	8.5 ± 1.3^{c}	2.1 ± 0.3^{c}	224 ± 57^{f}	25.9 ± 8.2^{f}
Fish (n = 25)	Baseline	9,499 ± 2,421	49.7 ± 6.1	16.6 ± 2.0	2.8 ± 3.9	31.0 ± 5.0	14.9 ± 3.2	11.2 ± 2.2	4.9 ± 1.5	224 ± 70	25.7 ± 10.7
	Week 1	9,539 ± 2,024	73.0 ± 3.6^{c}	12.2 ± 2.0^{c}	2.4 ± 3.1	12.4 ± 2.4^{c}	2.6 ± 0.4^{c}	7.6 ± 1.8^{c}	2.2 ± 0.3^{c}	14 ± 12^{c}	38.1 ± 12.9^{c}
	Week 3	8,786 ± 1,872	66.7 ± 4.0^{c,f}	15.1 ± 2.1^{c,f}	2.1 ± 2.4	16.1 ± 2.3^{c,f}	3.6 ± 0.5^{c,f}	7.5 ± 1.4^{c}	5.0 ± 0.6^{c,f}	80 ± 22^{c,f}	25.6 ± 7.8^{f}

Values are mean ± SD. Abbreviations: Carbohy. = carbohydrate; Sat. = saturated fatty acids; Mono. = monounsaturated fatty acids; Poly. = polyunsaturated fatty acids. ^{a,b,c}Significantly different to baseline ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$). ^{d,e,f}Significantly different to Week 1-vegetarian ($^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$)

the intake of LC PUFA during the white meat, red meat, and fish dietary periods are shown in Table 3. The amounts consumed daily were 231 ± 52 g of white meat (range 150–270 g white meat for females and 205–330 g white meat for males), 351 ± 104 g of red meat (range 255–400 g red meat for females and 330–500 g red meat for males), 133 ± 32 g of fish (range 90–155 g fish for females and 115–200 g fish for males). The mean 20:4n-6 intake was approximately 77 mg/day during the baseline period, and this almost doubled during the meat and fish diet phases of the study (Table 3). The 20:5n-3 levels of about 50 mg/day on the baseline diet declined significantly on the white meat phase and increased by a factor of 16 in the fish period. The DHA values of about 100 mg/day fell significantly on the meat diets to less than 40 mg/day and increased to about 2050 mg/day on the fish diet.

Significant changes in serum PL fatty acid concentration occurred for only six fatty acids during the study (Table 4). Oleic acid (18:1) levels increased during the vegetarian week of the white and red meat phases, which coincided with the highest consumption period of 18:1 by subjects. After 2 wk on each diet, the 18:1 levels decreased, particularly during the fish phase. Linoleic acid (LA, 18:2n-6) decreased during each vegetarian stage and remained low after two weeks of white meat, red meat or fish consumption. Arachidonic acid increased significantly during the white meat and red meat phases of the study, but decreased during the fish phase.

Eicosapentaenoic acid levels decreased during the vegetarian week of each phase of the study. During the 2 wk of white meat consumption, the level of 20:5n-3 did not change; however, a small but significant increase occurred during the 2 wk of red meat consumption and a large increase occurred during the fish phase of the study. Docosapentaenoic acid (22:5n-3) remained unchanged during the vegetarian week of each phase, but increased during the 2 wk of red meat and fish consumption. DHA (22:6n-3) was only significantly elevated during the fish phase of the study. As anticipated from dietary composition, the serum ratio of 20:4n-6/20:5n-3 changed substantially during the course of the study. During the vegetarian stages, the average ratio was 11:1; this increased to 15:1 on the white meat diet (poor in 20:5n-3) then decreased to 8:1 on red meat and 2:1 on the fish diet.

The changes in platelet fatty acids were relatively minor compared with the serum PL, apart from substantial increases in 20:5n-3 and 22:6n-3 after the fish phase (Table 5). The level of stearic acid (18:0) in the platelet PL decreased significantly during the fish phase of the study ($P < 0.001$), while LA decreased in the final 2 wk of each study. During the white and red meat phases of the study, 20:4n-6 levels remained unchanged, but fell significantly during the fish phase.

TABLE 3
Estimated Daily Intake of Long-Chain Fatty Acids (mg/day) During the Three, Two-Week Periods of Meat and Fish Consumption, Compared with the Mean Baseline Intake Levels Prior to the Study[a]

		Long-chain polyunsaturated fatty acid intake							
		AA[b]		EPA[c]		DPA[d]		DHA[e]	
	Meat (g)	Baseline	Diet	Baseline	Diet	Baseline	Diet	Baseline	Diet
White meat	231 ± 52	78 ± 32	140 ± 33[f]	51 ± 44	1 ± 0	33 ± 18	19 ± 7	109 ± 102	39 ± 9
Red meat	351 ± 104	77 ± 32	137 ± 34	51 ± 45	70 ± 19	33 ± 18	73 ± 19	107 ± 105	18 ± 7
Fish	133 ± 32	76 ± 35	138 ± 33	49 ± 46	847 ± 207	32 ± 21	487 ± 119	108 ± 105	2047 ± 499

[a]Values are means ± SD.
[b]Arachidonic acid, 20:4n-6, [c]Eicosapentaenoic acid, 20:5n-3.
[d]Docosapentaenoic acid, 22:5n-3, [e]Docosahexaenoic acid, 22:6n-3.
[f]Arachidonic acid intake kept constant on each phase of the study.

TABLE 4
Serum Phospholipid Fatty Acid Concentrations (mg/100 mL Serum) During Each Study

		Serum phospholipid fatty acid concentration					
Study	Stage	18:1	18:2n-6	20:4n-6	20:5n-3	22:5n-3	22:6n-3
White	Baseline	15.9 ± 2.5	30.3 ± 6.6	13.0 ± 3.5	1.5 ± 0.6	1.3 ± 0.3	4.7 ± 1.2
meat	Week 1	17.8 ± 3.4[c]	23.9 ± 4.5[c]	13.5 ± 3.9	1.2 ± 0.5[c]	1.7 ± 1.7	5.0 ± 1.6
(*n* = 23)	Week 3	17.4 ± 4.1[c]	23.8 ± 4.2[c]	16.0 ± 4.1[c,f]	1.1 ± 0.7[c]	1.3 ± 0.3	5.3 ± 1.2[a]
Red	Baseline	15.4 ± 3.3	29.1 ± 7.9	13.3 ± 4.1	1.5 ± 0.6	1.2 ± 0.3	4.8 ± 1.7
meat	Week 1	16.4 ± 3.8[b]	22.5 ± 5.0[c]	13.7 ± 3.9	1.2 ± 0.4[b]	1.2 ± 0.3	5.3 ± 1.8[b]
(*n* = 23)	Week 3	15.4 ± 2.9[e]	22.4 ± 5.6[c]	16.5 ± 5.0[c,f]	2.0 ± 0.8[f]	1.5 ± 0.5[b,e]	5.1 ± 1.8[b]
Fish	Baseline	15.0 ± 2.7	30.7 ± 7.5	14.2 ± 4.0	1.9 ± 1.0	1.4 ± 0.4	5.1 ± 1.4
(*n* = 25)	Week 1	15.9 ± 3.1	24.3 ± 5.8[c]	13.7 ± 3.8	1.3 ± 0.5[b]	1.4 ± 0.4	5.3 ± 1.4
	Week 3	12.8 ± 3.1[c,f]	18.5 ± 4.9[c,f]	12.0 ± 2.9[c]	6.5 ± 2.2[c,f]	1.7 ± 0.6[b,e]	13.3 ± 3.7[c,f]

Values are mean ± SD. No change was observed in the concentration of 14:0, 16:0, 16:1, 18:0, or 18:3n-3 fatty acids.
[a,b,c]Significantly different to baseline ([a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$).
[d,e,f]Significantly different to week 1—vegetarian ([d]$P < 0.05$, [e]$P < 0.01$, [f]$P < 0.001$).

TABLE 5
Platelet Phospholipid Fatty Acid Composition (percentage of total fatty acids) During Each Study

		Platelet phospholipid fatty acid composition						
Study	Stage	18:0	18:1	18:2n-6	20:4n-6	20:5n-3	22:5n-3	22:6n-3
White	Baseline	17.3 ± 2.0	14.3 ± 1.4	6.2 ± 0.8	27.5 ± 3.1	0.7 ± 0.2	1.9 ± 0.4	2.1 ± 0.3
meat	Week 1	16.8 ± 2.3	15.1 ± 2.1	5.5 ± 0.7[c]	26.8 ± 4.4	0.5 ± 0.2[c]	1.7 ± 0.4	2.2 ± 0.3
(*n* = 23)	Week 3	16.1 ± 1.6	16.1 ± 2.1[b]	5.3 ± 0.6[c,f]	27.1 ± 2.7	0.4 ± 0.1[c,f]	1.5 ± 0.3	2.2 ± 0.3
Red	Baseline	14.7 ± 2.0	14.6 ± 1.1	6.3 ± 0.9	29.6 ± 2.8	0.7 ± 0.2	1.9 ± 0.4	2.3 ± 0.4
meat	Week 1	14.3 ± 1.7	15.4 ± 1.3[b]	5.7 ± 0.7[c]	29.8 ± 3.1	0.5 ± 0.2[c]	1.8 ± 0.3	2.3 ± 0.4
(*n* = 23)	Week 3	14.3 ± 1.9	15.7 ± 1.2[b]	5.5 ± 0.5[c]	29.3 ± 2.4	0.7 ± 0.2[f]	1.9 ± 0.3	2.1 ± 0.3[b,e]
Fish	Baseline	16.8 ± 1.4	15.4 ± 0.9	6.3 ± 0.8	27.0 ± 2.4	0.6 ± 0.1	1.9 ± 0.3	2.0 ± 0.3
(*n* = 25)	Week 1	16.1 ± 1.0[c]	16.4 ± 1.2[c]	5.6 ± 0.6[c]	27.1 ± 2.1	0.4 ± 0.1[a]	1.7 ± 0.3	2.0 ± 0.3
	Week 3	15.5 ± 1.1[c,f]	16.9 ± 1.1[c]	5.1 ± 0.5[c,f]	24.1 ± 2.1[c,f]	1.9 ± 0.5[c,f]	1.8 ± 0.2	3.9 ± 0.5[c,f]

Values are mean ± SD. No change was observed in the concentration of 14:0, 16:0, 16:1, or 18:3n-3 fatty acids.
[a,b,c]Significantly different to baseline ([a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$).
[d,e,f]Significantly different to week 1—vegetarian ([d]$P < 0.05$, [e]$P < 0.01$, [f]$P < 0.001$).

During each vegetarian period, 20:5n-3 levels decreased significantly; however, the 20:5n-3 level increased significantly during the red meat and fish phases. Platelet levels of 22:6n-3 decreased during the red meat phase and doubled during the fish phase of the study. No significant change was detected in the platelet PL ratio of 20:4n-6/20:5n-3 during the white or red meat diets. However, the fish diet resulted in a decrease from 45:1 (baseline diet) to 13:1.

There were no significant changes in platelet aggregation (data not shown) on any of the studies. However, when all baseline data for the three studies were grouped and compared with the grouped vegetarian data, a significant but unexplained increase in aggregation due to collagen was observed after 1 wk of low-fat vegetarian diet relative to baseline (baseline 64.0% ± 2.4%, vegetarian 71.0% ± 2.3%, $P < 0.01$).

Using collagen as an agonist (Table 6), the *ex vivo* platelet TXB_2 production increased during the vegetarian period of the white meat study ($P < 0.05$) and returned to baseline following 2 wk of white meat consumption ($P < 0.05$). During the vegetarian weeks of the white meat and fish studies, the

TABLE 6
Thromboxane B_2 Produced by Platelets During Aggregation, Using the Agonists Collagen (C), Thrombin (T)

Study	Stage	Platelet TXB_2 (C) (ng/mL)	Platelet TXB_2 (T) (ng/mL)
White	Baseline	10.6 ± 2.2	4.6 ± 3.3
meat	Week 1	12.1 ± 1.5[a]	7.0 ± 2.5[b]
(*n* = 23)	Week 3	10.6 ± 1.1[d]	6.7 ± 3.6[a]
Red	Baseline	7.9 ± 2.2	5.2 ± 3.4
meat	Week 1	8.5 ± 2.8	6.2 ± 3.4
(*n* = 23)	Week 3	8.8 ± 1.5	7.2 ± 3.7
Fish	Baseline	9.1 ± 2.2	5.5 ± 3.2
(*n* = 25)	Week 1	9.6 ± 2.4	6.6 ± 3.2[a]
	Week 3	9.3 ± 2.4	5.1 ± 3.5[d]

[a,b,c]Significantly different to baseline ([a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$).
[d,e,f]Significantly different to week 1—vegetarian ([d]$P < 0.05$, [e]$P < 0.01$, [f]$P < 0.001$). Values are mean ± SD.

TABLE 7
Urinary Prostacyclin and Thromboxane Metabolite Levels Measured as a Total Daily Excretion

	White meat (n = 23)	Red meat (n = 23)	Fish (n = 25)
Daily PGI_2-M excretion (ng/day)			
Baseline	445 ± 198	442 ± 188	397 ± 130
Week 1	345 ± 157[c]	373 ± 213	333 ± 140[b]
Week 3	339 ± 140[c]	410 ± 187	326 ± 108[b]
Daily TXA_2-M excretion (ng/day)			
Baseline	898 ± 422	804 ± 305	807 ± 358
Week 1	834 ± 276	873 ± 396	834 ± 297
Week 3	851 ± 340	937 ± 367	715 ± 263[d]

Week 1 = end of vegetarian diet; week 3 = end of meat or fish diet.
[a,b,c]Significantly different to baseline ([a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$).
[d,e,f]Significantly different to Week 1—vegetarian ([d]$P < 0.05$, [e]$P < 0.01$, [f]$P < 0.001$).
Values are mean ± SD.

thrombin-stimulated platelets showed an increased TXB_2 production ($P < 0.01$ and $P < 0.05$, respectively). This decreased during the 2 wk of fish consumption ($P < 0.05$), but remained elevated on the white meat diet.

The daily urinary excretion of PGI_2-M decreased during the 1-wk vegetarian period of each study, reaching significance in the white meat ($P < 0.001$) and fish studies ($P < 0.01$) and remaining depressed, relative to baseline values, during the 2 wk of white meat ($P < 0.001$) and fish consumption ($P < 0.01$) (Table 7). No significant change in PGI_2 output was recorded during the 2 wk of meat or fish consumption relative to the first-week low-fat vegetarian diet. Prostacyclin (PGI_3), derived from the n-3 PUFA 20:5n-3, was detected in most subjects following 2 wk of fish consumption only, and reached a mean value 12 ± 9 ng/day.

The daily urinary excretion of TXA_2-M showed large interindividual variation, with no significant changes during the vegetarian week of each study. No effect of white or red meat consumption was evident, but a significant reduction occurred during the 2 wk of fish consumption ($P < 0.05$) relative to the vegetarian diet.

DISCUSSION

In the Western diet, the main dietary PUFA is linoleic acid, and this fatty acid is almost certainly the major source of the 20:4n-6 which is found in high proportions in most cell membranes of the body (34). High intakes of fatty fish or fish oil rich in LC n-3 PUFA are known to increase membrane PL levels of these LC n-3 PUFA, with such diets often leading to simultaneous reductions in the 20:4n-6 levels in the membranes. These changes have been associated with beneficial effects on thrombosis tendency, including reduced TXA_2 production, reduced platelet aggregation, and lowered plasma triglyceride levels (35).

We have been interested in the converse situation, that is, what are the consequences of ingesting diets containing 20:4n-6 (21,30). In the early 1960s Mohrhauer and Holman (36) showed that on a gram for gram basis, dietary 20:4n-6 led to substantially higher tissue 20:4n-6 levels than dietary LA. The purpose of the present study was to examine the effects of the ingestion of a constant dietary level of 20:4n-6 combined with varying levels of LC n-3 PUFA (supplied from meat or fish) on serum and platelet PL 20:4n-6 levels and subsequent conversion to vasoactive eicosanoids in studies of two weeks' duration. By using white meat, red meat, and fish, we were able to supply a constant level of dietary 20:4n-6 at double the usual intake for these subjects and a varying LC n-3 PUFA intake (from very low to very high).

The meat diets were associated with approximately 20% increases in the serum PL 20:4n-6 concentration and little or no change in n-3 PUFA levels; however, there were no changes in the platelet 20:4n-6 levels on these diets in the short-term period of this study. In contrast, the fish diet led to very large increases in 20:5n-3 and 22:6n-3 in both plasma and platelet PL and to significant decreases in 20:4n-6 levels in both tissues. An obvious explanation for the changes in the platelet lipids on the fish diet (but not on the meat diets) was the increase in the dietary intake of n-3 PUFA by about 3 g/day compared with the increase in 20:4n-6 of only 60–70 mg/day on the meat diets.

There were no effects from any of these diets on *ex vivo* platelet aggregation. This is not surprising since the platelet PL 20:4n-6 on the fish diet was still 12 times the 20:5n-3 level, compared with Eskimos, where the levels of 20:4n-6 and 20:5n-3 in platelet lipids are about the same (37).

However, the fish diet was associated with a significant reduction ($P < 0.05$) in *ex vivo* TXB_2 production from thrombin-stimulated platelets and in the principal urinary metabolite of thromboxane (11-dehydro-TXB_2, TXA_2-M). This decrease in TXA_2-M daily excretion, following the 2 wk of fish consumption, coincided with a decrease in platelet PL 20:4n-6, an increase in 20:5n-3 and 22:6n-3, and a change in the 20:4n-6/20:5n-3 ratio in platelet PL from 68:1 (vegetarian diet) to 13:1. These changes could account for the 20% decrease in TXA_2 production by a number of mechanisms, firstly, replacement of 20:4n-6 in PL by LC n-3 PUFA, effectively lowering the amount of substrate for TXA_2 production

(38); secondly, inactivation of cyclooxygenase by LC n-3 PUFA (36); finally, release of 20:5n-3 from platelet PL, which competes with 20:4n-6 for access to cyclooxygenase and produces an alternative form of thromboxane (TXA_3) (39). Decreases in TXA_2 following fish consumption have been observed in other studies. Knapp and FitzGerald (40), found a 29% reduction in TXA_2 production, measured as the urinary metabolite 2,3-dinor-TXB_2, following 50 mL/day of fish oil for 4 wk. A small amount of TXA_3 was detected (~14% of the total thromboxane level). Ferretti *et al.* (41) observed a 38% reduction in TXA_2 production, measured as the urinary metabolite, 11-dehydro-TXB_2, following 10 wk of fish oil consumption (15 g/day). A small quantity of the TXA_3 metabolite was also detected, but not quantified. In the 2 wk of fish consumption in the present study, no TXA_3 products were detected, possibly owing to the lower level of 20:5n-3 consumed (~847 mg/day), compared with ~9 g/day and 4.5 g/day in the above two studies, respectively. Like many other studies involving fish oil consumption, the intake of LC n-3 PUFA the above two studies is at a pharmacological level and has little resemblance to normal human nutrition. It is likely that TXA_3 is not normally produced in humans consuming a modern Western diet even when modest amounts of fish are consumed, as in the 2-wk fish diet of the present study. White and red meat consumption did not result in a substantial change in platelet 20:4n-6/20:5n-3 ratio or TXA_2 production, even though both these diets significantly increased the 20:4n-6 concentration of the plasma PL. Presumably the low intake of 20:4n-6 and the short time frame of the study contributed to the failure to alter platelet 20:4n-6 levels. However the study was designed to test the impact of high 20:4n-6 intake from foods, in this case specifically meats. The limiting factors proved to be the actual physical quantity of meat that subjects could comfortably consume on a daily basis and the period of time that they could maintain this level of consumption.

The decrease in PGI_2-M recorded during the low-fat vegetarian period of each study is consistent with observations by Nordoy *et al.* of decreased prostacyclin production on low-fat diets (42), the suggested reason being that fatty acids (saturates in particular) stimulate PGI_2 production by endothelial cells. An *in vitro* study by Zhang *et al.* (43) showed stearic acid to increase PGI_2 production by endothelial cells in culture medium, but this increase was associated with morphological signs of cellular damage and reduced viability in culture. No further changes in PGI_2-M production were observed after the 2 wk of meat or fish diets. Previous studies have shown conflicting effects of LC n-3 PUFA on *in vivo* PGI_2 production. Mann *et al.* (32) showed a decreased level of urinary PGI_2-M in rats following 2 wk of dietary supplementation with 20:5n-3, and Ferretti *et al.* (31,41), in a study involving the feeding of 15g/day of fish oil to human subjects, found a 20% decrease in urinary PGI_2-M. In other studies, no change in urinary PGI_2-M was observed when fish oil or fish was fed to human subjects (38,44,45). Prakash *et al.* (45) found a decrease in TXA_2-M in subjects fed a similar diet to this study, containing 450 g/day of salmon (rich in 20:4n-6 and LC n-3 PUFA). In two other human studies involving fish consumption (46,47), urinary PGI_2-M levels were increased. However, it should be noted that in these two studies the level of 20:4n-6 contained in the fish consumed was high (~250 mg/day and ~380 mg/day, respectively). In the present study, a small amount of PGI_3-M (~4% of total prostacyclin) derived from 20:5n-3 was measured in the urine following 2 wk of fish consumption. Knapp and FitzGerald (40), feeding 50 mL/day of fish oil for 4 wk, also found an increase in the PGI_3-M level (from zero to ~15% of the total prostacyclin production).

In conclusion, it can be stated that under the circumstances of this study, there was no increase in TXA_2 production on diets containing 20:4n-6 and relatively low levels of n-3 PUFA (white and red meat), but there was a decrease on a fish diet (100–200 g/day), containing 90–210 mg/day 20:4n-6 and approximately 3 g of LC n-3 PUFA. However, this does not rule out the adverse effects of 20:4n-6 at higher levels in the diet or for more prolonged periods. The LC n-3 PUFA from fish had a beneficial effect on platelet reactivity by lowering *in vivo* TXA_2 production without significantly affecting PGI_2 production; however, the maintenance of PGI_2 levels on fish diets may be dependent on the simultaneous consumption of 20:4n-6, which is relatively high in salmon (14) compared with other sources such as white or red meat, and certainly supplies more 20:4n-6 than is present in fish oil feeding studies. White and red meat consumption within the ranges used in this study does not appear to change prostacyclin production; however, the low-fat diets did decrease prostacyclin production, and future studies will have to take this phenomenon into account.

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Noninvasive Characterization of Neonatal Adipose Tissue by ^{13}C Magnetic Resonance Spectroscopy

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ABSTRACT: *In vivo* ^{13}C magnetic resonance spectroscopy (MRS) was applied noninvasively to analyze the fatty acid composition of adipose tissue in 21 full-term newborn infants and 6 mothers. In order to assess the effects of gestational and postnatal age on adipose tissue composition, we studied preterm infants at birth, term infants at the ages of 6 wk and at 6 mon. We also investigated the influence of maternal diet on infant adipose tissue composition by studying the breast-fed infants of women who maintained either an omnivore or a vegan diet. Significant differences were observed in adipose tissue composition of neonates compared with their mothers. Neonates had more saturated and less unsaturated fatty acids than their mothers ($P < 0.01$). We also observed changes in adipose tissue composition with maturity. From birth to 6 wk of age ^{13}C MR spectra showed a significant increase in the amount of unsaturated fatty acids, particularly polyunsaturated fatty acids ($P < 0.01$). Similarly, differences were seen as a result of gestational age. Preterm infants had relatively fewer unsaturated fatty acids than full-term infants. A greater proportion of these unsaturated fatty acids were polyunsaturated. Our results demonstrate that ^{13}C MRS can be utilized to assess noninvasively neonatal adipose tissue lipid composition and to monitor the effects of developmental changes due to gestational age and oral feeding.
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Infancy is characterized by rapid growth and development. Neonatal adipose tissue fatty acids serve not only as an important energy reserve during this period but also as a source of lipid precursors for membrane development of organs such as the liver and brain (1–3). Preterm and low-birth-weight neonates have been shown to have considerably smaller reserves of adipose tissues, sometimes as little as 10% of that found in term neonates (2). The overall amount of fat the neonate carries appears to be important, and these small reserves may result in retarded growth, reduced ability to maintain body temperature, and impaired neurodevelopment (4). In addition, it has been suggested that the type of fatty acids available is of particular significance to membrane growth and the adipose tissue stores of polyunsaturated fatty acids may be essential for brain development (3).

Neonatal nutrition is thought to have an important influence on certain diseases in adult life, including cardiovascular disease, non-insulin dependent diabetes mellitus, and obesity (5,6). These studies have focused on the effect of birth weight. However, it is known that the fatty acid composition of adipose tissue in adults is an independent risk factor for cardiovascular disease (7). This raises the question of whether the type of fatty acids present in the neonatal adipose also affects adult disease development.

Examination of the fatty acid composition of subcutaneous adipose tissue of infants has so far required tissue samples obtained by biopsy or at postmortem for *in vitro* analysis by gas–liquid chromatography (GLC) (8–14). It has therefore not been possible to perform serial, longitudinal studies of adipose tissue composition of healthy infants. A noninvasive technique would permit the investigation of the relationship between neonatal nutrition, adipose tissue composition, and subsequent growth and development.

Recently magnetic resonance spectroscopy (MRS) has been applied to the study of lipids in humans (15–18) and animals (19–21). Although individual fatty acids cannot be identified, Moonen *et al.* (15) have shown that from an *in vivo* ^{13}C MR spectrum of human adipose tissue, it is possible to determine the quantities of saturated and unsaturated fatty acids present (15). Beckmann *et al.* (16) used ^{13}C MRS to show differences in the adipose tissue profile of subjects after 6 mon dietary change, and we have previously shown that ^{13}C MRS can demonstrate long-term dietary-dependent differences in adipose tissue composition (17).

The objectives of this study were to noninvasively assess, by ^{13}C MRS, differences in the adipose tissue composition of healthy full-term newborn infants and their mothers. Further, we determined the influence of gestational age and the effect of oral feeding on adipose tissue composition of neonates.

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Abbreviations: GLC, gas–liquid chromatography; MRS, magnetic resonance spectroscopy; S/N, signal-to-noise ratio; TPN, total parenteral nutrition.

MATERIALS AND METHODS

Volunteer assessment. Permission for this study was obtained from the Ethics Committee of the Royal Postgraduate Medical School, Hammersmith Hospital, London (No. 93/4237). Healthy full-term infants (n = 21, 12 males, 9 females), with a mean gestational age of 38.7 ± 0.3 wk (± SEM) (range 37–42 wk) and a mean birth weight of 3.3 ± 0.2 kg (range 2.0–5.1 kg), were studied as soon as possible after birth (mean study day after birth 1.8 ± 0.4, range 0–7 d). The mothers (all omnivores) of six of the infants were also studied on the same day.

Ten infants (4 male, 6 female) had a further examination at the age of 6 wk (mean age 6.2 ± 0.5 wk, range 4–10 wk), eight of whom had received breast milk and two were formula fed. Two of these infants (both male) were reexamined at the age of 6 mon. In addition, three vegan infants (all female, mean age 6.7 mon) and their mothers were studied. All of the 6-mon-old infants had been wholly breast-fed by their mothers. Infants were partially weaned to a vegan or omnivore diet at the time of the study.

Spectra were also obtained from six preterm infants (all female) with a mean gestational age of 33.8 ± 0.5 wk (32–36 wk) and birth weight of 2.0 ± 0.1 kg (1.7–2.6 kg). One preterm baby (gestational age 26 wk, birth weight 1.0 kg, weight at examination 1.9 kg) who had been exclusively fed by total parenteral nutrition (TPN) was studied at the age of 5 wk.

^{13}C MRS. In vivo ^{13}C MR data were obtained at 1.5 T on a Picker prototype system. Spectra were collected from the adipose tissue of the left thigh from the mother and from the upper thigh and buttocks of the neonates. An enveloping saddle transmitter coil and a surface receiver coil (14 or 6 cm) were used. Shimming was performed on the proton water signal in the preterm infants, but in the full-term infants and their mothers, shimming was possible directly on the ^{13}C lipid signals. Each examination lasted approximately 15 min and no sedation was required as infants were studied during natural sleep. During each examination infants were observed with MRS-compatible ECG and pulse oximetry monitors.

Coupled ^{13}C MR spectra of adipose tissue were acquired using a 90° pulse. In initial studies, T_1 and signal height ratio measurements were obtained from adults and neonates (22). Spectra for quantitative analysis were obtained with eight acquisitions at TR 30 s. Because of low signal-to-noise (S/N) ratio, spectra from the preterm infants were also obtained at a TR 300 ms with 256 acquisitions. The spectra were then analyzed using a correction factor to overcome partial saturation effects. This factor was obtained from the ^{13}C MR spectra of full-term infants by measuring the peak areas of several spectra acquired at different repetition times (TR 300 ms and TR 30 s) and calculating the change in the spectra. The factor obtained from the full-term infants was then applied to the spectra obtained from the preterm infants at TR 300 ms for them to be analyzed quantitatively.

Data analysis. Spectral analyses were carried out using a modified Microcal Origin software package (Microcal Software, Inc.) in combination with biochemical prior knowledge, since standard commercially available software could not easily be used. The free induction decays were zero filled to 4 K prior to Fourier transform, and no line broadening was used. The *in vivo* ^{13}C MRS examination introduces some intrinsic difficulties, which need to be considered during data analysis. The S/N can often be limited in infant subjects. Furthermore, the ^{1}H-^{13}C long-range spin-spin couplings (more than one bond apart) are similar to the resonance half-linewidths and cannot be resolved at the low magnetic field strength used, complicating the spectral lineshape. We found therefore that the Gaussian lineshape given by the equation

$$G_i = \frac{A_i}{a_i \times \sqrt{\pi/2}} \times \exp\left(\frac{-2 \times (\nu - \nu_i)^2}{a_i^2} \right) \quad [1]$$

(where A_i is the area of the resonance, a_i the half-linewidth, ν the spectral frequency and ν_i the resonance frequency of the Gaussian i) described the experimental lineshapes much more closely than the Lorentzian shape. In addition, incorporation of biochemical prior knowledge of the lipid resonances into the spectral analysis was found to be advantageous, especially in the analysis of spectra from infant subjects. In particular, use of prior knowledge decreased the noise-related variability of the analysis and increased the overall reliability of the results (23–26). All analyses included a linear baseline $c_0 + c_i\nu$.

^{13}C MR spectra from 98 adult volunteers were pooled and the carbonyl and unsaturated resonances analyzed using one Gaussian component for each resonance. The pooled data showed that the relative chemical shifts of the resonances were unchanged from person to person. Therefore, all the analyses for the current data were performed by first separately analyzing the carbonyl resonance (at around 172 ppm) and then the unsaturated lipid resonances (from 110 to 129 ppm) keeping the resonance frequencies fixed relative to the carbonyl chemical shift (–46.8 and –48.6 ppm from the carbonyl resonance frequency for the mono- and polyunsaturated and polyunsaturated resonances, respectively). By examining the spectra from all of the adults and from the nine infants who had spectra with sufficient S/N ratio, we were able to obtain a reliable individual estimation of the half-linewidths. The relative chemical shifts of the resonances in the spectra from the nine infant subjects were the same as those obtained for the pooled adult data set. However, the resonances in the spectra from the infant subjects were somewhat broader and less well resolved than the corresponding resonances in the spectra from the adult volunteers. Therefore, based on the analyses of the spectra with good S/N ratio from the nine infants, average values for the resonance half-linewidths were calculated and used in the analysis of all other spectra from infants (19.9 Hz for the carbonyl, 19.1 Hz and 19.5 Hz for the mono- and polyunsaturated and polyunsaturated resonance, respectively). Inclusion of this kind of prior knowledge guaranteed unequivocal analysis and resulted in calculated spectra that closely resembled the experimental data (Figs. 1 and 2).

Total fat analysis. Total unsaturated and saturated fatty acids were calculated (Method A) as previously described

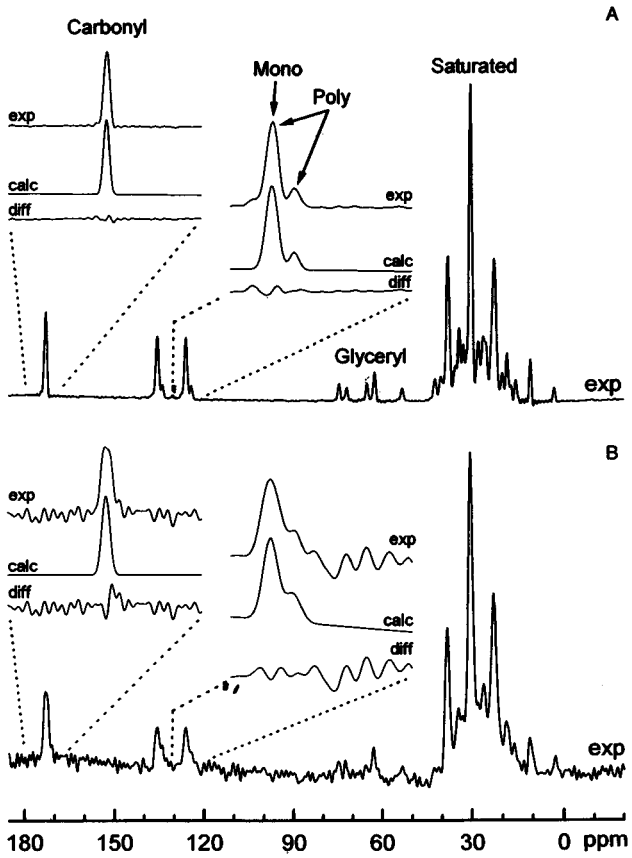

FIG. 1. *In vivo* ^{13}C magnetic resonance spectra of adipose tissue from a (A) mother and her (B) full-term neonate (TR = 30 s). Resonances arising from the carbonyl, unsaturated, glyceryl, and saturated regions of triglycerides may be observed. Assignments: carbonyl carbons 172.1 ppm; carbons in the monounsaturated (mono) and polyunsaturated (poly) positions (–**C**H=**C**H– and –***C***H=CH–CH$_2$–CH=***C***H–) 134.9 and 125.4 ppm; polyunsaturated carbons only (–CH=***C***H–CH$_2$–***C***H=CH–) 133.4 and 124.2 ppm; carbons from glycerol backbone 60–70 ppm; carbons in the saturated position (–***C***H$_2$– or –***C***H$_3$) 10–40 ppm. The results of the lineshape-fitting analysis illustrating the experimental (exp), calculated (calc), and difference (diff) spectra for the carbonyl and unsaturated resonances are also shown (for more details see the Materials and Methods section).

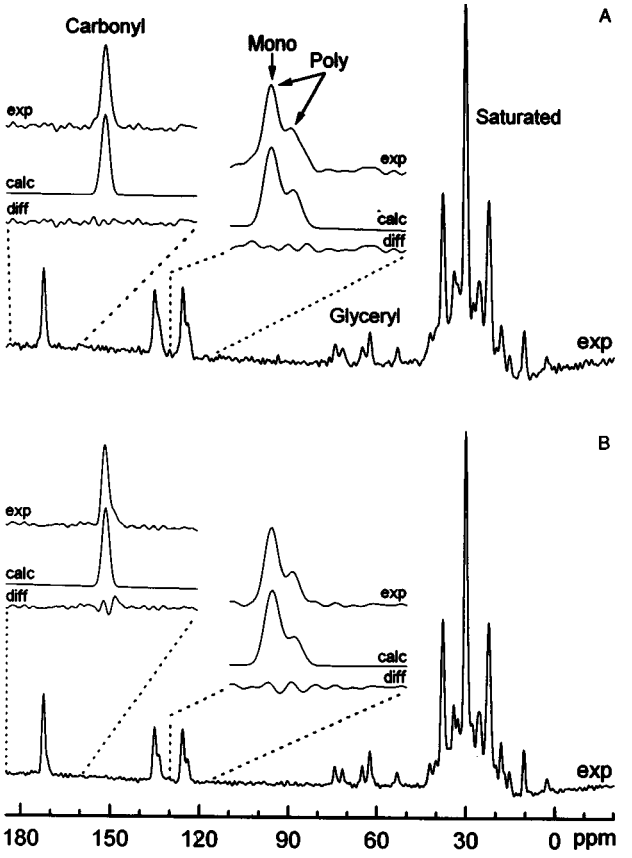

FIG. 2. ^{13}C magnetic resonance spectra from the adipose tissue of 6-mon-old infants breast-fed by (A) vegan and (B) omnivore mothers. For a full assignment, description, and abbreviations, see Figure 1.

(15,16). This method assumes that linoleic acid is the major polyunsaturated fatty acid in adipose tissue. However, this fatty acid is not nearly so prominent in infant adipose tissue as in adults (8–14) and for this reason the method of percentage carbon analysis (Method B) was also used (17,20,22). Both methods are described briefly below.

Total fat analysis by Method A: This method allows the calculation of total levels of polyunsaturated, monounsaturated, and saturated fatty acids (15), allowing direct comparison with literature values obtained by GLC.

$$\%\ \text{polyunsaturated} = \frac{\left(I_p + I_p\right)}{2I_{\text{C=O}}} \times 100 \qquad [2]$$

$$\%\ \text{monounsaturated} = \frac{\left(I_{M+P} + I_{M+P}\right) - \left(I_P + I_P\right)}{2I_{\text{C=O}}} \times 100 \qquad [3]$$

$$\%\ \text{saturated} = 100 - (\%\ \text{polyunsaturated} + \%\ \text{monounsaturated}) \qquad [4]$$

where $I_{C=O}$ = intensity of carbonyl peak (172.1 ppm); I_{M+P} = intensity of monounsaturated (M) and polyunsaturated (P) coupled resonances (134.9 and 125.4 ppm); I_P = intensity of polyunsaturated coupled resonances (133.4 and 124.2 ppm).

Percentage fatty acid carbon analysis by Method B: This method is based on absolute data, makes no assumptions about the composition of the tissue under study, and overcomes the problems of analyzing the unsaturated region of the ^{13}C MR spectra. Percentages of carbons in saturated (found in both saturated and unsaturated fatty acid chains) and unsaturated (found only in unsaturated fatty acid chains) positions in the fatty acid chain are calculated from integrals of three regions of the ^{13}C spectrum: carbonyl groups at 165–175 ppm; unsaturated fatty acid carbons at 120–140 ppm; and saturated fatty acid carbons at 10–45 ppm. This method involved the summing of integrated areas of the carbonyl, unsaturated, and saturated regions of the spectrum to provide a total fatty acid area:

$$\%\ \text{total fatty acid carbons}\ (A) = I_{\text{carbonyl}} + I_{\text{unsaturated}} + I_{\text{saturated}} \qquad [5]$$

$$\%\ \text{unsaturated fatty acid carbons}\ (B) = \frac{I_{\text{unsaturated}}}{A} \times 100 \qquad [6]$$

$$\% \text{ saturated fatty acid carbons} = 100 - B \quad [7]$$

The unsaturated region of the ^{13}C MR spectrum has two pairs of resonances: (i) the larger peaks (I_{M+P}) at 134.9 and 125.4 ppm arise from carbons in the monounsaturated and polyunsaturated positions of the fatty acid chains (–**C**H=**C**H– and –**C**H=CH–CH_2–CH=**C**H–); (ii) the smaller peaks (I_P) at 133.4 and 124.2 ppm arise from carbons in the polyunsaturated positions of the fatty acid chain (–CH=**C**H–CH_2–**C**H=CH–). The proportion of fatty acid carbons that were polyunsaturated or monounsaturated may be determined from the following equations:

$$\% \text{ polyunsaturated fatty acid carbons} = \frac{I_P + I_P}{(I_P + I_P) + (I_{M+P} + I_{M+P})} \times B \quad [8]$$

$$\% \text{ monounsaturated fatty acid carbons} = B - \% \text{ polyunsaturated} \quad [9]$$

Statistical analysis. Differences between the term, preterm, and adult groups were examined using Student's unpaired *t*-test. Differences between the term infants examined at birth, 6 wk and 6 mon later were tested using the Student's paired *t*-test. All results presented are expressed as mean ± SEM.

RESULTS

Representative ^{13}C MR spectra obtained *in vivo* from the adipose tissue of a full-term infant at birth and his mother are shown in Figure 1. Separate resonances arising from carbon atoms from different regions of the triglyceride chains can be observed in both spectra and include: carbonyl (–**C**=O), unsaturated (–**C**=**C**–), glyceryl (–**C**H_2– and –**C**H–), and saturated fatty acid carbons (–**C**H_2– and –**C**H_3). There are clear differences in the relative intensity of saturated and unsaturated fatty acids. Full-term infants at birth had significantly more saturated fatty acids than their mothers [58.4 ± 1.7 vs. 17.9 ± 2.9%, $P < 0.01$] (Method A, see Table 1) and conversely fewer unsaturated fatty acids [41.6 ± 1.7 vs. 82.1 ± 2.9%, $P < 0.01$]. By 6 wk of age, spectra from these infants had changed, showing increases in unsaturated fatty acids [59.6 ± 1.4 vs. 41.6 ± 1.7%, $P < 0.01$], though not to the level found in their mothers.

TABLE 1
Fatty Acid Composition of Adipose Tissue of Mothers, Preterm Infants, and Full-Term Infants at Birth and After 6-wk Development, Analysis Using Method A[a]

	Preterm ($n = 6$)	Full-term at birth ($n = 21$)	6-wk-old infants ($n = 10$)	Mothers ($n = 6$)
Unsaturated (%)	38.9 ± 1.9	41.6 ± 1.7	59.6 ± 1.4[b]	82.1 ± 2.9[a]
Saturated (%)	61.1 ± 1.9	58.4 ± 1.7	40.4 ± 1.4[b]	17.9 ± 2.9[a]

[a]Mean ± SEM. [a]$P < 0.01$, level of significance between mothers and full-term infants at birth. [b]$P < 0.05$, level of significance between full-term infants at birth and 6 wk later.

By expressing the fatty acid content of adipose tissue as percentage fatty acid carbons (Table 2), similar differences can again be observed. This method allows quantification of poly- and monounsaturated fatty acids without making assumptions as to the type of fatty acids present (17). Saturated and unsaturated fatty acid carbons were significantly different in full-term infants compared with their mothers. Lower levels of both polyunsaturated and monounsaturated fatty acid carbons were observed in full-term infants compared with their mothers. The spectra from full-term infants at 6 wk showed an increase in unsaturated fatty acid carbons (infant at birth: 7.5 ± 0.2 vs. 6-wk-old infant: 8.5 ± 0.3%), becoming closer in appearance to that of their mothers. Two infants had a further examination at the age of 6 mon. No significant differences were found between the adipose tissue composition of the infants at the age of 6 wk vs. 6 mon (unsaturated, 6 wk: 8.5 ± 0.3 vs. 6 mon: 8.7 ± 0.8%). However, the adipose tissue of the infants at 6 mon was still significantly different from their mothers with lower levels of unsaturated fatty acid carbons.

Preterm infants had slightly higher relative levels of saturated fatty acid carbons and lower unsaturated fatty acid carbons than full-term infants. When the signal from unsaturated fatty acid carbons was further analyzed for the relative levels of mono- and polyunsaturated fatty acids, preterm infants had lower relative levels of monounsaturated fatty acid carbons than their full-term counterparts (Table 2). Higher levels of polyunsaturated fatty acid carbons were found in the adipose

TABLE 2
Percentage Fatty Acid Carbons Found in the Adipose Tissue of Mothers, Preterm Infants and Full-Term Infants at Birth and After 6 wk Development, Analysis Using Method B[a]

	Preterm ($n = 6$)	Full-term at birth ($n = 21$)	6-wk-old infants ($n = 10$)	Mothers ($n = 6$)
Unsaturated (%)	5.9 ± 0.6[c]	7.5 ± 0.2	8.5 ± 0.3[b]	10.5 ± 0.3[a]
Saturated (%)	93.7 ± 0.6	92.4 ± 0.2	91.5 ± 0.3	89.5 ± 0.3
Polyunsaturated (%)[d]	2.4 ± 0.3	2.1 ± 0.1	2.3 ± 0.2	2.7 ± 0.3
Monounsaturated (%)[e]	3.5 ± 0.3[c]	5.4 ± 0.2	6.1 ± 0.3[b]	7.8 ± 0.2[a]

[a]Mean ± SEM. [a]$P < 0.01$, level of significance between mothers and full-term infants at birth. [b]$P < 0.05$, level of significance between full-term infants at birth and at 6 wk. [c]$P < 0.01$ level of significance between full-term and preterm infants at birth. [d]Polyunsaturated refers to carbons in –CH=***C***H–CH_2–***C***H=CH– positions. [e]Monounsaturated refers to carbons in –***C***H=***C***H and –***C***H=CH–CH_2–CH=***C***H– positions.

TABLE 3
Comparison of Adipose Tissue Composition of 6-mon-old Infants Breast-Fed by Vegan and Omnivore Mothers, Analysis Using Method B

	Vegan infants (n = 3)	Omnivore infants (n = 2)
Unsaturated (%)	11.0 ± 0.3	8.7 ± 0.8
Saturated (%)	89.0 ± 0.3	91.3 ± 0.8
Polyunsaturated (%)	4.3 ± 0.4	2.5 ± 0.4
Monounsaturated (%)	6.7 ± 0.1	6.2 ± 0.4

TABLE 4
Comparison of Adipose Tissue Composition of 6-mon-old Infants Breast-Fed by Vegan Mothers and Their Mothers, Analysis Using Method B

	Vegan infants (n = 3)	Vegan mothers (n = 3)
Unsaturated (%)	11.0 ± 0.3	12.1 ± 0.2
Saturated (%)	89.0 ± 0.3	87.9 ± 0.2
Polyunsaturated (%)	4.3 ± 0.4	4.3 ± 0.1
Monounsaturated (%)	6.7 ± 0.1[a]	7.8 ± 0.2

[a] $P < 0.01$, level of significance between vegan infants and their mothers.

tissue of the preterm compared to the term infants, although this did not reach the level of significance. The adipose tissue fatty acid composition of the preterm infant receiving TPN closely resembled that of the TPN formula (results not shown).

Representative spectra obtained from the adipose tissue of vegan and omnivore infants at 6 mon are shown in Figure 2. The adipose tissue composition of infants breast-fed by vegan mothers were shown to have higher levels of unsaturated fatty acid carbons in their adipose tissue than infants fed by omnivore mothers [11.0 ± 0.3 vs. 8.7 ± 0.8%] (see Table 3).The vegan infants also had higher levels of polyunsaturated fatty acids than the omnivore infants [4.3 ± 0.4 vs 2.5 ± 0.4%]. No significant differences were found in the polyunsaturated fatty acid composition of adipose tissue composition between the vegan mothers and their infants (Table 4).

DISCUSSION

There is a paucity of information regarding the effects of maternal and postnatal nutrition on neonatal adipose tissue composition and its association with development. This arises from a lack of methods for the assessment of neonatal body composition other than invasive procedures such as biopsy. Until now, investigating noninvasively the effect of diet on adipose tissue composition in healthy infants has not been possible, so that detailed longitudinal studies have not yet been performed. This preliminary study shows that natural abundance ^{13}C MRS allows the noninvasive and repeated measurement of the fatty acid composition of adipose tissue of newborn infants. Using ^{13}C MRS, we showed differences between newborn infants and mothers and showed the influence of gestational age and postnatal maturity on adipose tissue composition of newborn infants.

This is the first study of *in vivo* ^{13}C MRS in newborn infants. No comparative data are therefore available. However, our data may be compared with *in vitro* GLC analysis of tissue samples from preterm and full-term neonates. Published values for the fatty acid composition of adipose tissue in both preterm (saturated 37.9–57.8%, unsaturated 42.0–55.7%) and term infants (saturated 50.8–57.6%, unsaturated 42.8–49.2%) show a large variation (8–14). This probably reflects variability in patient population and methodology (8–14). Our measurements are similar to the range given in the GLC literature, suggesting that the ^{13}C MRS measurements reflect the composition of neonatal adipose tissue.

We have observed clear differences in adipose tissue fatty acid composition between mothers and full-term newborn infants. At birth, newborn infants had significantly more saturated and correspondingly fewer unsaturated fatty acids than their mothers. These differences may arise from the fact that during fetal life glucose is the major substrate for *de novo* synthesis of fatty acids, leading mainly to the production of saturated fatty acids. Polyunsaturated fatty acids predominantly arise directly from maternal sources across the placenta. The higher levels of polyunsaturated fatty acids found in the mother provide the necessary gradient for these essential fatty acids to be transported to the developing fetus (10). This gradient is clearly maintained throughout the gestational period, and the magnitude of this gradient may determine the final level of polyunsaturated fatty acid in the neonate. This appears to suggest that the levels of polyunsaturated fatty acids in the maternal adipose tissue may determine the final levels of these fatty acids in newborn babies. Our preliminary results in the study of vegan mothers and their newborn infants suggest that this may be the case. Further work is underway to determine the relationship between maternal adipose tissue, diet, and neonatal adipose tissue composition.

Marked changes were observed in the ^{13}C MR spectra of the infants who were studied again after 6 wk. The proportion of saturated fatty acids had decreased and that of unsaturated fatty acids increased, resulting in spectra similar to those obtained from their mothers. These changes predominantly reflect the effects of oral feeding on adipose tissue composition. Previous GLC studies have suggested that the fatty acid composition of neonatal adipose tissue changes rapidly during postnatal development and is particularly influenced by milk fat composition (8–14). The adipose tissue composition of the 6-wk-old infants had not altered significantly at the time of the repeated examination at the age of 6 mon. This would suggest that growth and postnatal development had little effect on adipose tissue composition during this period. This finding is in agreement with previous GLC studies which showed little change in the fatty acid composition of adipose tissue in milk-fed infants beyond the age of 1 mon (9,11–13).

Gestational age is known to have a profound effect on the composition of adipose tissue (2,9,10,13). This is clearly confirmed in this study. We found significant differences in the

^{13}C MR spectra of preterm and full-term newborn infants. Since all these infants were examined immediately following birth, the differences observed arise from gestational age differences rather than postnatal external factors such as diet. Preterm infants had more saturated and fewer unsaturated fatty acids than full-term infants. Also, the proportion of polyunsaturated and monounsaturated fatty acids differed. These findings are similar to those previously reported on samples of adipose tissue from infants of differing maturity (2,9,10,13). Accretion of polyunsaturated fatty acids during the third trimester of pregnancy is particularly rapid (2). Previous studies have suggested that during the last trimester of pregnancy there is a 12-fold increase in adipose tissue stores, while total body weight increases only 3-fold. It is thought that only a few additional weeks of intrauterine development will result in the different fatty acid composition of adipose tissue seen between the preterm and term neonates (2). These differences can be clearly observed by *in vivo* ^{13}C MRS.

Preliminary data on infants fed by vegan mothers compared to infants fed by omnivore mothers show a clear trend. The adipose tissue composition of infants directly reflects that of their mothers, with the vegan infants having 70% more polyunsaturated fatty acid carbons than the omnivore infants. Although the consequences of essential fatty acid deficiency in formula-fed infants are well documented, less is known about the effects of very high levels of long-chain polyunsaturated fatty acids in the infant diet. The long-term effects for vegan infants having such high levels of polyunsaturated fatty acids in their diet and adipose tissue are unknown. It may be important to measure the dietary intake of antioxidants in these infants to ensure that rates of lipid peroxidation are not excessively high. Another related problem could be an excessively high n-6/n-3 ratio; competition between n-6 and n-3 long-chain polyunsaturated fatty acids may result in insufficient n-3 fatty acids in the infants of vegan mothers.

The results of this study show that *in vivo* ^{13}C MRS can be used to examine the effects of diet on adipose tissue composition and allows the possibility of using noninvasive methods to study changes due to maturity and to different feeding strategies.

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Thermotropic Phase Behavior of *in vivo* Extracted Human Stratum Corneum Lipids

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ABSTRACT: The thermotropic phase behavior of lipids extracted either *in vivo* from inner forearm (SCLE) or plantar callus (PC) was investigated by differential scanning calorimetry and small angle X-ray diffraction. PC composition was chromatographically modified (MPC) by eliminating the more polar lipids in order to evaluate their role. Analysis of composition confirms the potential use of PC as a source of stratum corneum lipids. MPC and SCLE exhibit similar differential scanning calorimetry (DSC) profiles with a main transition around 50°C attributed to the solid-to-liquid phase transition of the ceramides. The absence of a transition around 50°C for PC suggests the possible perturbation of ceramide packing by the significantly high proportion of phospholipids. X-ray data suggest a high miscibility of sebum components in stratum corneum lipids with possible modification of chain packing. The MPC patterns show a lipid phase separation which underscores the role of polar lipids in cholesterol/free fatty acids/sterol esters/ceramides structural cohesion.
Lipids 32, 653–660 (1997).

The stratum corneum (SC) controls the permeability of human skin and protects the organism against water loss and cutaneous penetration of deleterious environmental agents (1,2). Transport across sebaceous lipids which are widespread at the skin surface and in the SC is the rate-limiting step in the percutaneous absorption process. In this respect, the intercellular lipid pathway represents the major route of transport across the SC. Keratinocytes, the most abundant cells of the epidermis, synthesize the major structural components of the SC in a programmed differentiation process regulated in time and space (3). The SC (10- to 20-μm thick) is composed of many layers of flat cornified cells filled with keratin separated by a unique and complex mixture of lipids (4). These lipids have been analyzed and shown to vary with location, age, sex, season, and pathological state (5,6). Furthermore, the lipids in the epidermis present a composition gradient from the inner part of the body to the surface that is linked to the differentiation process. Lipids are released from the lamellar bodies during differentiation, and early studies have shown a shift from polar to neutral lipids during cornification. Lipids from adult SC are mainly composed of polar and nonpolar lipids, including phospholipids, cholesterol-3-sulfate, cholesterol, glycosylceramides, ceramides, and sterol esters. These lipids display a local organization, in particular due to ceramide 1, a 30-carbon ω-hydroxy acid amide linked to a sphingosine base bearing an ester-linked linoleic acid on the ω-hydroxy acid, covalently linked to the corneocyte envelope. Extracted intercellular lipids should present a different organization from the native state since they should exclude ceramide 1. Sebaceous lipids, widespread at the skin surface, are mainly composed of triglycerides (TG), wax esters, squalene, and some free fatty acids (FFA).

The thermotropic phase behavior and structural parameters of lipids constituting the intercellular cement of human SC have been a subject of investigation over the last decade. Such studies based on differential scanning calorimetry (DSC), infrared spectroscopy, or X-ray diffraction analysis can indeed provide important insight into the function of these lipids and their role in the interactions of the SC with foreign chemicals (7–13). The specimens used in these experiments consisted of either sheets or solvent-extracted lipids of SC isolated from plastic surgery or human cadaver skin. Other studies focused on reconstructed epidermis (14) or model membrane systems approximating the SC composition (15,16). There have been no reports involving the analysis of *in vivo* extracted human skin lipids.

We have recently proposed a nonirritating solvent system for the *in vivo* extraction of human SC lipids and developed a high-resolution method of lipid analysis by high-performance thin-layer chromatography (HPTLC) (17). This method allows extraction of lipids from the upper layers of the SC together with the sebum film lipids of the skin surface. Thus the method gives access to the lipids first encountered by topically applied molecules. The present study focuses on the organization of the extracted lipids which differs in nature from the native state. The ultimate aim of the work is to understand

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Abbreviations: DSC, differential scanning calorimetry; FFA, free fatty acids; HPTLC–AMD high-performance thin-layer chromatography–automated multiple development; MPC, modified plantar callus extract; PC, plantar callus; SC, stratum corneum; SCLE, stratum corneum lipid extract; TG, triglycerides.

the thermotropic behavior of human skin lipids in relation to their composition.

In the present paper, thermotropic transitions and structural characteristics of lipids from two distinct human sources, *in vivo* extracted SC (inner forearm) and plantar callus (PC) were investigated by DSC and X-ray diffraction. PC lipid composition was modified by a chromatographic method in order to study the relative importance of the various components. The results are discussed as a function of the molecular composition of the specimens.

MATERIALS AND METHODS

In vivo *extraction of SC lipids.* This study was conducted in the springtime in healthy volunteers free of dermatological diseases and with untanned skin (Caucasian males, $n = 11$, mean age 32.5 ± 7.5). Six neighboring zones of the inner forearm were chosen. Prior to extraction, the surface of each zone was stripped once with a 22-mm diameter adhesive disk, D-Squame (Cuderm Co., Dallas, TX) after application of a constant pressure (180 g per cm^2) and a constant time of 30 s. A 10-cm glass tube open at both ends, with an area of 3.14 cm^2, was pressed on the skin and filled with 5 mL of a nonirritating solvent to extract upper-layer SC lipids. The solvent used was cyclohexane/ethanol (2:8, vol/vol) as previously described (17). After 1 min of contact and gentle manual shaking, the solvent was transferred to a glass tube. The *in vivo* extraction was done twice and followed by two similar extractions with cyclohexane/ethanol (5:5, vol/vol). The SC lipid extracts (SCLE) were combined and dried under nitrogen.

Extraction of PC. Pooled PC specimens were collected from a chiropodist. After visual inspection for hair or nail contamination, a 10-g sample of callus was extracted three times with 100 mL hexane/methanol (2:3, vol/vol) under sonication in an ultrasonic bath (15 min,160 watts; Branson, Danbury, CT), filtered through a 0.45 µm HV filter (Millipore, Saint-Quentin, France), concentrated under vacuum to dryness, dispersed in a minimum of water, and freeze-dried to constant weight. This extract was called "PC."

Modified PC lipids mixture. The more polar lipid class, i.e., the phospholipids and some ceramides, were eliminated from the PC mixture by silica chromatography as follows: 110 mg of PC was solubilized in 102 mL of chloroform/methanol (95:5, vol/vol) sonicated for 10 min and divided into 6 aliquots of 17 mL. Each aliquot was purified on a 1 g Sep-Pack silica micro column (Millipore), previously conditioned with chloroform/methanol (95:5, vol/vol). For each fraction, the column was washed with 5 mL of chloroform/methanol (95:5, vol/vol) then washed twice with 5 mL of hexane. All the eluted solutions were pooled, dried under nitrogen, and stored at −20°C; 18.8 mg of product called modified plantar callus extract MPC, was obtained.

Lipid analysis. Lipid contents of the three pools SCLE, PC, and MPC were analyzed by HPTLC-automated multiple development (AMD) as recently described (17) except for TG and FFA which were separated and quantified by HPTLC. For TG and FFA analysis, a four-step elution was carried out: once with toluene, followed by three steps with hexane/diethyl ether/formic acid (80:20:0.4, by vol). Lipids were dissolved in cyclohexane/ethanol (35:65, vol/vol) (SCLE) or in hexane/methanol (40:60, vol/vol) (PC, MPC). Five microliters were automatically applied on HPTLC plate. After migration, plates were scanned at 450 nm (TLC 2 scanner, cats3 software; Camag, Basel, Switzerland) after visualization by spraying with 10% (wt/vol) cupric sulfate pentahydrate in 8% (vol/vol) *o*-phosphoric acid and heating for 10 min at 110°C for all lipids except for TG and FFA which were heated 30 min at 150°C (17). The limits of detection and lower point of calibration (in parentheses) were as follows: phospholipids 0.10 (0.2) µg, glycosylceramides 0.09 (0.1) µg, cholesterol-3-sulfate 0.10 (0.1) µg, ceramides 0.20 (0.2) µg, cholesterol 0.10 (0.1) µg, TG 10 ng (0.1 µg), FFA 1 ng (0.1 µg), wax esters 0.09 (0.1) µg, sterol esters 0.10 (0.1) µg, squalene 0.18 (0.2) µg.

DSC. The thermal behavior of lipid samples was studied with a DSC 4 apparatus (Perkin-Elmer, Norwalk, CT) from 5 to 60, 65, or 80°C, depending on the samples, at heating and cooling rates of +5°C/min and −5°C/min, respectively. All experiments were carried out in hermetically sealed aluminum pans. The lipids (from 2 to 3.5 mg dry weight) were first deposited in the pans in 50 µl of a solution of ethanol/cyclohexane (65:35, vol/vol) for SCLE, and methanol/hexane (30:20, vol/vol) for PC and MPC. The solvent was then removed by gentle evaporation under nitrogen before freeze-drying for 12 h. Hydration was performed by adding pure water directly in the pans before sealing to obtain a final water content of 60% by weight. The closed DSC vessels were stored for 3 d at 4°C until analysis.

X-ray diffraction. All experiments were performed on the D24 line of the DCI Synchrotron source at LURE (Orsay, France) by using the initial setup of P. Vachette and C. Bourgaux. The camera produces a highly collimated beam with a cross section nearly equal to 0.4×1 mm^2 at the sample position. The wavelength of the radiation was 1.49 Å. Data were collected by means of a 1024 channel Ar/CO_2- or Xe-filled linear detector. Cylindrical vacuum chambers equipped with capton or mylar windows were placed in the beam pathway to limit X-ray absorption by air. The sample-to-detector distance was set to either 28 or 142 cm for wide-angle or small-angle analysis, respectively. The diffraction patterns were normalized with respect to synchrotron-beam decay and acquisition times. The diffraction band observed at a position close to $q = 0.4$ $Å^{-1}$ is related to the capton window of the vacuum tube placed just after the sampleholder. Calibrations were performed by using tristearin with a repeat distance of 45 Å (18). All the experiments were performed in duplicate using independent samples of each lipid source.

Thin glass capillaries (GLAS, Müller, Berlin, Germany) were used as sample cells. The lipids were first introduced in solution in the same organic solvents as those used for DSC experiments. Solvents were removed under nitrogen flow followed by 12 h of freeze-drying. Final lipid dry weights of ap-

proximately 5 mg were deposited in the capillaries. The required amounts of water to obtain a rate of hydration of 60% by weight were directly added to the sample cells by successive low-speed centrifugations (not exceeding 1000 rpm). The capillaries were then sealed under argon to avoid both water loss and sample oxidation and the lipid–water mixtures were placed in a thermostated bath at 40°C for 1 h. Samples were stored at 4°C until X-ray measurements.

For measurements, the capillaries were placed in a specially designed thermal controller holder developed by G. Keller *et al*. (19). Samples were heated by means of an electric wire. Temperature was measured by a thermocouple introduced near the sample cell and regulated by the use of both the Peltier effect for cooling and the Joule effect for heating. The samples were equilibrated for 5 min at fixed temperatures at which the diffraction patterns were recorded. Between the successive temperature steps, the samples were heated at a rate of 1°C/min.

The scattering intensities are plotted as a function of q which is linked to the scattering angle θ and the wavelength λ by the equation $q = 4\pi \sin\theta/\lambda$. The Bragg law gives the distance d of the repeating units ($n\lambda = 2d \sin\theta$), q being $2\pi/d$ and n an integer.

RESULTS

Lipid composition. Table 1 reports the HPTLC–AMD comparative analysis of three samples: SC lipid extracts originating from inner forearm (SCLE), PC, and MPC, respectively.

SCLE contains ceramides and cholesterol in addition to a few phospholipids and sterol derivatives, i.e., the characteristic lipids of SC as well as squalene, wax esters, and TG which are recognized as the major constituents of sebum (4,5,8,17). Moreover, the TG and FFA contents are in agreement with those previously reported for face origin (4). It is worth noting that the sebum contamination is not negligible, although inner forearm is known to be almost exempt from sebaceous units, and sebum secretion at this site is low (20).

The composition of PC shows only SC lipids and is close to Melnik's published results except that the phospholipid content in our sample is higher probably due to the chiropodist source (21). The absence of sebum components is in agreement with the anatomical origin of this sample. Also, the results indicate that PC is naturally devoid of TG. With respect to the SCLE sample, it follows that these lipids are mostly due to sebum components in the *in vivo* extracted sample.

MPC was prepared from PC by eliminating the more polar compounds, phospholipids, and some of the ceramides. This is explained by the fact that the resolution of the flash chromatographic method does not currently allow the discriminating separation of these two classes of compounds.

DSC profiles. Figure 1, curves a–c, shows the differential calorimetric scans of hydrated SCLE, PC, and MPC, respectively. DSC scans are rather noisy owing to extraction and loss of the native organization, but peak occurrence is not doubtful since a correlation to X-ray recordings as a function of temperature is observed. The phase transitions of the three samples occur over a broad temperature range. SCLE (Fig. 1, curve a) and MPC (Fig. 1, curve c) undergo two low-energy endothermic transitions, a first one in the 20–35°C range and a predominant one in the 40–60°C range, while PC (Fig. 1,

TABLE 1
Lipid Composition of Biological Extracts

Lipids	SCLE[a] (wt%)	PC[b] (wt%)	MPC[c] (wt%)
Phospholipids	5.2	17.6	n.d.[d]
Glycosylceramides	n.d.	n.d.	n.d.
Cholesterol-3-sulfate	1.0	Trace[e]	n.d.
Ceramides	16.0	32.4	26.5
Cholesterol	11.0	32.4	44.2
Triglycerides	13.7	Trace	Trace
Free fatty acids	17.5	11.7	17.3
Wax esters	23.5	n.d.	n.d.
Sterol esters	5.0	5.9	9.6
Squalene	7.1	Trace	2.4
Total	100	100	100

[a]SCLE: stratum corneum lipids extracted *in vivo* on the inner forearm of male volunteers.
[b]PC, plantar callus lipid extract.
[c]MPC, modified PC extract. Each lipid is expressed as the percentage of total lipid extract (wt%).
[d]n.d., not detected.
[e]Trace, detected but under the lowest limit of linearity (see Ref. 17).

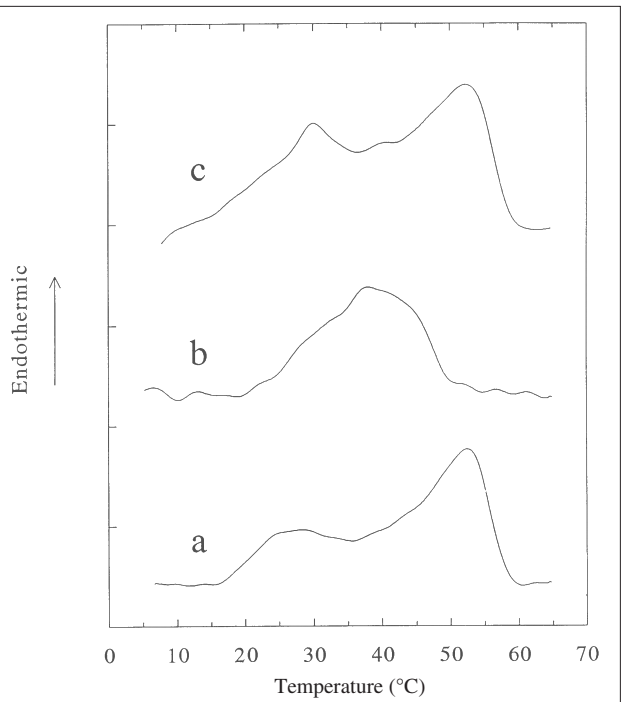

FIG. 1. Differential scanning calorimetry (DSC) thermograms of (a) *in vivo* extracted stratum corneum lipids (stratum corneum lipid extract), (b) plantar callus, (c) modified plantar callus. The samples were hydrated at 60% (by weight), and scans were performed with a heating rate of 5°C/min. After heating at 65°C, cooling at 5°C, and keeping the samples at this temperature for 3 d, reheating led to the same DSC profiles, indicating the reversibility of the endothermic transitions.

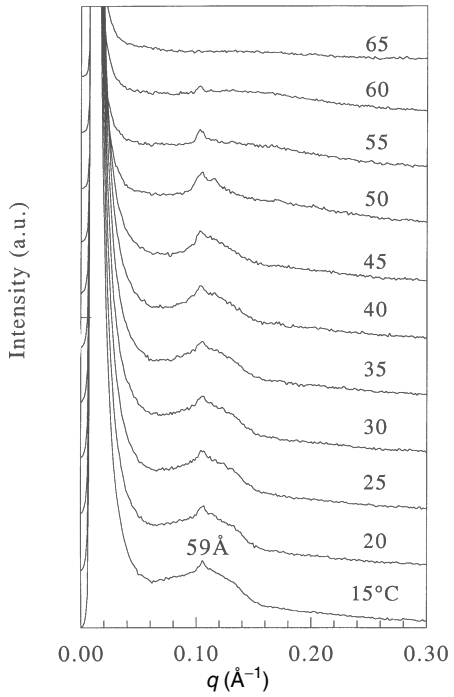

FIG. 2. Small-angle X-ray diffraction patterns of hydrated (60% by weight) *in vivo* extracted stratum corneum lipids (stratum corneum lipid extract) as a function of temperature. Sample heating was performed at 1°C/min. X-ray patterns were recorded at fixed temperatures, every 5°C, after a 5-min equilibrium time. The resolved characteristic distance is reported on the first pattern. The intensities are reported in arbitrary units by using the same scale for each pattern.

curve b) apparently shows only one endotherm over the 25–50°C range. On cooling of the three samples, only one exotherm was observed which corresponds to the principal transition. After 3 d at 4°C, all the transitions were observed again on heating, making the system reversible with a presumably long equilibrium setting time.

X-ray diffraction. The scattering curves recorded as a function of temperature are shown in Figures 2–4. For SCLE and PC the broad and relatively weak diffraction features are related to the presence of a very complex mixture composed of nine classes of lipids (Table 1). For SCLE (Fig. 2) at temperatures below 50°C, a broad scattering band is observed on top of which a main diffraction peak is located at q = 0.106 Å^{-1}. The corresponding repeat distance is 59 Å. As the sample is heated, the scattering signal first progressively disappears between 45 and 55°C while the main diffraction peak is still observed at 60°C. At 65°C all diffraction bands have disappeared.

For PC (Fig. 3), a main diffraction peak appears at q = 0.066 Å^{-1} (distance of 95 Å) with probably its second-order at q = 0.133 Å^{-1}. A shoulder on the right side of the peak is also present at q = 0.08 Å^{-1}. In the 20–35°C temperature range, both peaks at 0.066 and 0.133 Å^{-1} disappear and only the shoulder remains. This shoulder is progressively replaced above 45°C by a broad diffraction peak at q = 0.06 Å^{-1} (105 Å), the intensity of which increases from 40 to 60°C. At 70°C, the remaining diffraction peak has almost totally vanished.

For MPC (Fig. 4A) five diffraction peaks were principally observed at low temperature. The peaks at q = 0.048 Å^{-1} and 0.096 Å^{-1} correspond to the first and second orders of a repeat distance of 129 Å. The peaks at q = 0.188 Å^{-1} and 0.375 Å^{-1} are related to the same Bragg spacing of 33.5 Å. The fifth peak is detected at q = 0.149 Å^{-1} (43 Å). In addition, another diffraction band can be seen at very small angles but is not resolved since it is mainly superimposed with the rings of the direct beam. The evolution of the different diffraction peaks is visualized as a function of temperature in Figure 4B. In the 15–40°C range, the peaks corresponding to periods of 129 Å and 43 Å, respectively, show similar slight increases in intensity. This very likely implies that the peak at d = 43 Å mainly corresponds to the third order of the main repeat distance of 129 Å. From 40 to 50°C, the peak at d = 129 Å progressively decreases in intensity and disappears above 50°C. In contrast, the intensity of the peak at 43 Å significantly increases. This strongly suggests that in the 40–65°C temperature range, the lamellar structure of period 129 Å is disappearing while a new distinct lipid organization (peak at 43 Å) is emerging.

DISCUSSION

There are several published reports on the X-ray determination of the structure of the human SC isolated from either trypsinized plastic surgery specimens or, more recently, from

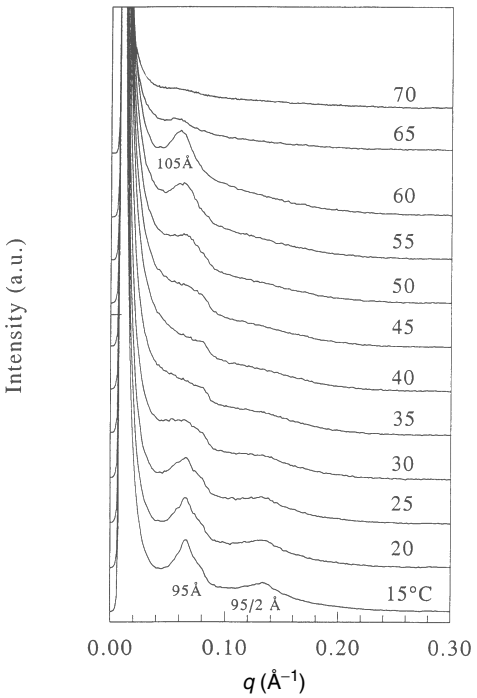

FIG. 3. Small-angle X-ray diffraction patterns of hydrated (60% by weight) plantar callus lipids as a function of temperature. See Figure 2 for experimental details.

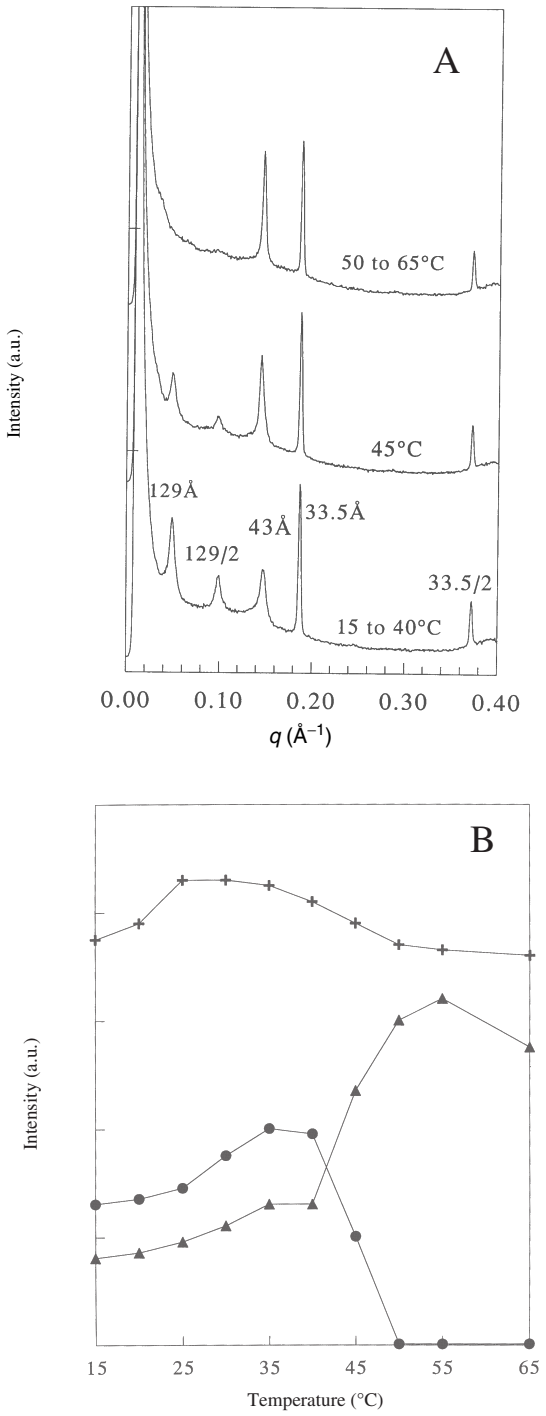

FIG. 4. A) Small-angle X-ray diffraction patterns of hydrated (60% by weight) modified plantar callus lipid extract (MPC) as a function of temperature. The sample was prepared by eliminating the more polar lipids from plantar callus. See Figure 2 for experimental details. (B) Variations of the scattered intensity of hydrated MPC as a function of temperature for the different diffraction peaks observed at $q = 0.048$ Å^{-1} ($d = 129$ Å; ●), $q = 0.148$ Å^{-1} ($d = 42$ Å; ▲) and $q = 0.188$ Å^{-1} ($d = 33.5$ Å; ◆).

dermatomed skin of patients with lamellar ichthyosis (9,10,12,22). Entire sheets were examined with or without lipid extraction (mainly with chloroform/methanol mixtures) in order to study the influence of the different compartments (lipids and corneocytes) in SC organization. DSC studies on similar specimens have also been reported (7–9).

The present study focused on the thermal and structural behavior of the main components of the lipids between corneocyte layers. These lipids are indeed known to modulate the skin barrier properties. First, extractions were performed either *in vivo* (SCLE) or from PC, which is an abundant source of SC. Second, the PC sample was modified in order to gain further information on the role of the different constituents in the SC structure.

Analysis of SCLE lipids shows that this sample is similar in composition to that of SC lipid extracts reported in the literature, providing that the sebum contaminants are not taken into account (4,5,17,21). In this respect, lipid analysis confirms that most of the TG found in SCLE originate from sebum. In contrast, for PC and MPC, the high proportion of phospholipids found in the first sample and the elimination of the more polar lipids from the second one lead to compositions which do not completely reflect human SC extract, despite its usually noted variability. Nevertheless, comparison of SCLE, PC, and MPC should provide information about the effect of sebum, as SCLE is contaminated while PC and MPC are not, and about the effect of phospholipid proportions on the structural properties of the extracted intercorneocyte lipids.

The DSC recording presented for SCLE is in agreement with previously published findings on SC lipids of human origin (7,8,10). Nevertheless, only one high-energy transition has been found around 55°C in SCLE while in native SC, two transitions are found at 65 and 75°C, respectively. In fact, the second transition was attributed to the nonextractable lipids of SC since they are linked to the proteins of the corneocyte membrane (7,8,10). Lowering of the main transition temperature to 55°C instead of the usually reported value of 65°C may be explained by the difference of the extraction methods. The procedures commonly described consist in incubation of entire sheets of SC in organic solvent such that the recovered extracts are claimed to contain the totality of the extractible lipids. In the case of *in vivo* extraction, the solvent used may reach the first intercellular layers of the SC, whose composition may differ from that of the deeper layers (23). MPC and SCLE lipid mixtures exhibit similar thermal behaviors in particular showing a negligible influence of sebum up to 44% contamination. This can be related to the previous results obtained with addition of 1% of sebum (8). The main transition observed around 40–60°C for SCLE and MPC can be ascribed to the solid-to-liquid phase transition of ceramides (24). For PC, the absence of an endothermic transition near 50°C coincides with the tripling of the phospholipid content in relation to SCLE. This suggests that a high proportion of phospholipids may affect ceramide packing. In addition, the significant width of the PC endotherm raises the possibility that the main transition observed with the other two samples (SCLE, Fig. 1 curve a ; MPC, Fig. 1 curve c) may also exist for PC (Fig. 1 curve b) but would be shifted toward lower temperatures, because of the high phospholipid content. Thus,

both transitions observed for SCLE and MPC would merge in the case of PC. The low-energy endotherm (20–35°C) characterizing the SCLE and MPC samples has already been attributed to the orthorhombic to hexagonal transition of the lipid chain packing (8,25,26). In the case of PC, Figure 1 shows that this transition is also observed.

The results of the small-angle X-ray diffraction analysis reveal the role of the individual lipid classes in the structural order. The asymmetry of the diffraction bands found below 20°C for SCLE (Fig. 2) and PC (Fig. 3) may indicate the probable existence of more than one single-phase arrangement. However, no significant lipid segregation is observed. In particular, no visible sebum phase separation appears. The structure of PC, for which a second-order Bragg spacing is detected, probably related to a lamellar structure, appears more organized than SCLE. This can be related to sebum components in SCLE that also contribute to diffraction peak spreading and that decrease the main repeat distance from 95 to 59 Å. Sebum compounds can therefore be incorporated to a high extent in the extracted skin lipids, possibly by inducing lipid chain interpenetration. One can speculate that a similar phenomenon may occur in SC. According to this hypothesis, modification of lateral chain packing should be involved and should directly influence the permeation properties of the skin. MPC patterns show a lipid phase separation, i.e., the coexistence of a lamellar structure of large period (129 Å) very likely governed by ceramides, cholesterol (33.5 Å), and a third structure with a Bragg spacing of 43 Å, presumably owing to fatty acids which present such typical repeat distances (27). Similarly, phase separation has already been demonstrated for cholesterol/ceramide mixtures (27–29). These results taken together underscore the strong contribution of polar lipids to the cohesion of the SC extracted lipid structure. Indeed, it has been recently shown that mixtures of cholesterol and ceramides form lamellar arrangements without cholesterol segregation when equimolar proportions are used (30). Moreover, considering that the polar lipids eliminated from PC mainly include phospholipids (Table 1), these last should also play an important role in cholesterol insertion and interact with the ceramide matrix to modify the period of their lamellar structure from 129 Å (MPC) to 95 Å (PC). This supports the hypothesis, already suggested by DSC experiments, that phospholipids can shift the main endotherm to lower temperatures by affecting ceramide packing.

Comparison of the DSC and X-ray analyses shows that disappearance or modification of the lamellar structures occurs at the temperatures corresponding to the offset of the main transition observed in DSC: 60°C for SCLE and MPC, and 50°C for PC. In particular, results concerning SCLE agree with data previously reported for SC collected by scraping (22), confirming that the *in vivo* extracted SCLE constitutes a reliable SC model. Interestingly, regardless of the extracted pool, the lipid organization, probably governed by the ceramides, is preserved below 35°C (Figs. 2,4). Consequently, the secondary thermal transition depicted at low temperature apparently does not affect the period of the lamellae. In contrast, the wide-angle X-ray diffraction patterns recorded as a function of temperature show that the offset of the low energetic transition (approximately 40°C) coincides with the loss of the chain packing order. Indeed, the short spacing observed at 4.15 Å (q = 1.514 Å^{-1}) progressively disappears with heating from 15 to 35°C and vanishes at 40°C. Figure 5 illustrates the behavior for PC. It is worth noting that the X-ray data support the hypothesis mentioned above for PC that there exist two effective thermal events, similar to SCLE and MPC. The first event corresponds to the fusion of the lipid chains in the 20–35°C range, and the second predominant one leads to the disappearance of the principal lamellar distance of 95 Å in the 40–50°C range. In addition, the X-ray diffraction pattern in Figure 3 shows the formation at 50°C of a new molecular organization with a characteristic distance of 105 Å which disappears above 65°C. However, no thermal event is recorded in this temperature range by DSC. Such a phase transition may result in the sum of exothermal and endothermal events such that no signal is detected in this temperature range. This might also be explained by the different thermal treatments (heating rates, equilibrium times at fixed temperatures) used for DSC and X-ray analyses, respectively, wherein DSC conditions may be too fast to allow the formation of this transient structure with a layer spacing of 105 Å.

In conclusion, the innovation of the present work consists in the *in vivo* extraction of the lipids from the superficial lay-

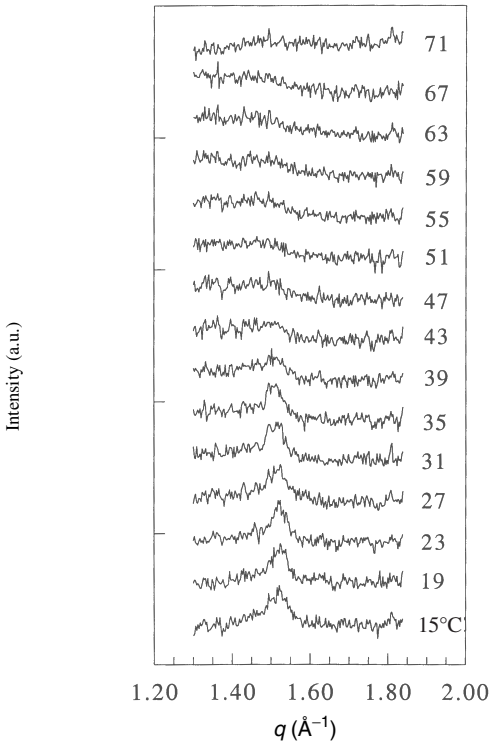

FIG. 5. Wide-angle X-ray diffraction patterns of hydrated (60% by weight) plantar callus lipids as a function of temperature. Sample heating was performed at 1°C/min. X-ray patterns were recorded at fixed temperatures, every 4°C after a 5-min equilibrium time. The intensities are reported in arbitrary units by using the same scale for each pattern.

ers of human SC and a combination of quantitative chemical analysis, DSC, and X-ray diffraction in order to elucidate the structural properties of these lipids in their hydrated state in relation to their nature and degree of association. Composition analysis shows that the use of nonirritating solvents results in extraction of high amounts of sebum contaminants, i.e., wax esters, TG, and squalene, together with the actual intercorneocyte lipids, mainly ceramides and cholesterol derivatives. In contrast, plantar callus lipid extract does not show sebum contamination. Both samples are characterized by a unique molecular arrangement governed by ceramides and which is presumably lamellar, indicating that sebum components are miscible with SC lipids by inducing chain interpenetration. The elimination of phospholipids from the PC extract leads to a lipid phase separation that demonstrates the essential role of polar lipids in ensuring a cooperative molecular arrangement of the cholesterol/FFA/sterol esters/ceramides mixture. PC should represent an easily available source to constitute a reliable model for superficial SC lipid extract without sebum contamination, providing phospholipid content is controlled.

Our data have interesting implications regarding the interactions of topically applied molecules with skin. Since sebaceous lipids constitute nearly half of the *in vivo* extract, a molecule applied on the skin has an equal probability of encountering sebum and/or SC lipids. The high miscibility between sebum and intercorneocyte lipids raises the possibility of *in vivo* contamination of SC superficial layers by sebum. This may be particularly relevant when considering that the probable entanglement of the lipids constituting the SC caused by sebum could enhance the permeability of the SC layer. Thus, the understanding of skin penetration of a molecule in the outer SC layers should involve the study of the interactions of this molecule not only with sebum and SC lipids considered separately but also with a mixture of both. In addition, the skin penetration of a pharmacological compound is governed by its affinity for sebum and/or SC lipids, as a function of the anatomical site of delivery. Comparison of our results with the literature led us to conclude that MPC is indeed the only mixture that contains the long periodicity (130 Å) lamellar phase, very characteristic of the native SC lipid organization. Moreover, the possibility evoked in this work of differentiating lipid phases by controlling the amount of phospholipids may make it possible to explore fine interactions between guest molecules (e.g., drugs, penetration enhancers, excipients) and the different SC components. Our findings also raise the question of the influence of exogenous topically applied lipids on the SC structural order. To this end, experiments on the SC thermotropic phase behavior will be carried out in the presence or absence of sebum using various model compounds.

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The Relationship Between the Structure of Monoalkyl Branched Saturated Triacylglycerols and Some Physical Properties

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ABSTRACT: Three different physical properties, the gel point ("solidification point"), the refractive index and the density, were determined and related to the structure of the branched triacylglycerols. A four-factor central composite face-centered design was constructed where the four variables were the length of the main chain, the branching position, the length of the side chain, and the number of branched fatty acyl groups attached to the glycerol backbone. Second-order models were calculated in which the three physical properties were related to the structure. Four additional branched triacylglycerols were analyzed in order to confirm the validity of each model. Contour plots are shown in order to visualize the prediction equations which were obtained.

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Triacylglycerols containing branched fatty acids occur sparingly in nature. They are known components of, e.g., human sebum (1,2) and adipose tissue of lambs (3–5). At least in human sebum, the branched fatty acids occur more frequently in the 2-position than in the 1- and 3-positions (1). One important property of the branched triacylglycerols in human sebum seems to be its porosity for water vapor (6). Branched fatty acids also have been found in milk (7). The crystal structures of both branched saturated fatty acids and triacylglycerols have been described in the literature (8). Furthermore, esters between branched fatty acids and polyols, often others than glycerol, have found use in, e.g., lubricants, owing to excellent lubricity, stability toward oxidation, and low-temperature properties (9,10).

The physical properties of branched triacylglycerols have been investigated previously by Aydin *et al*. (11), who synthesized both the 1-monoacylglycerols and the homotriacylglycerols of nine different α-monoalkyl branched saturated fatty acids. The physical properties investigated were the refractive index (at 20°C), the dielectric constant, and the dipole moment.

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Abbreviation: ANOVA, analysis of variance.

However, no other systematic investigation has been carried out relating the physical properties of triacylglycerols containing monoalkyl branched saturated fatty acyl groups to their structure. In the present study the gel point ("solidification point"), the refractive index (at 60°C), and the density are related to the structure of such triacylglycerols. A chemometrical approach was applied to the problem resulting in a four-factor central composite face-centered design. The aim has been to cover all possible triglyceride structures with "normal" chain lengths (main chain between 6 and 18 carbons). This approach has required the syntheses of a number of new compounds (12). In the case of the gel point, any polymorphic properties which the triacylglycerols may have were not taken into consideration.

MATERIALS AND METHODS

Materials. All triacylglycerols have been synthesized (12). The objective has been to obtain high purities in order to minimize the effects of impurities on, e.g., the nucleation. The purity of the triacylglycerols was found to be >98% by gas–liquid chromatography and/or high-performance liquid chromatography. All compounds synthesized are racemic.

Measurements. The gel points were determined on a Bohlin VOR Rheometer (Lund, Sweden) used in the oscillation mode. The samples were placed between a cone and a plate with diameters of 25 mm and a gap size of 0.15 mm. The gap was adjusted at 0°C for the samples with high gel points (>−10°C) and at −25°C for the samples with low gel points (<−10°C). The torsion bar used had a torque constant of 92.3 gcm. The samples were subjected to an oscillation (frequency 0.500 s^{-1}) in autostrain mode. This means that the strain was adjusted according to the measured torque in such a way that increased torque leads to a decrease in strain. The strain amplitude varied between 0.002 and 0.2. The measurements were performed in a standard low-temperature cell cooled by liquid nitrogen. The samples were cooled from −10 to −100°C (10 to −10°C for the triacylglycerols with high gel points) with a temperature gradient of −0.5°C/15 s. The results were recorded every 6 s.

The gel points were determined by means of the tangent to the sharply increasing storage modulus (G′). This indicates an increase in the elastic component of the sample. The results are strongly dependent on the temperature gradient chosen, owing to the fact that the triacylglycerols show different polymorphic behavior and nucleation rates. This method of determining the gel point is a modification of the method of Bohlin *et al.* (13).

The gel points of the triacylglycerols with very high gel points (>10°C) were determined simply by agitating 2 g of the triacylglycerol contained in a small bottle with a thermometer using the same frequency as in the rheometer. The samples were cooled by the surrounding air and when necessary with an ice-water mixture. The cooling was carried out in such a way as to achieve the same gradient as with the rheometer (–2°C/min).

Both methods of determining the gel point ("solidification point") described above have in common that any polymorphic properties, if present, are not considered. This should be taken into account when looking at the results. One of the triacylglycerols in the present study, which had a gel point of 10°C, was a liquid at room temperature for more than 1 wk before crystallizing. As is the case with all methods concerning crystallization and melting (e.g., differential scanning calorimetry and rheology), the results depend on the exact method used and how the results are evaluated.

The refractive indices were determined at 60°C with an Abbé refractometer (Carl Zeiss, Jena, Germany), model A.

The densities were determined at 60°C with an Anton Paar Precision density meter (Instrument AB Lambda, Sollentuna, Sweden), OMA 02C.

Experimental design. The structures of the monoalkyl branched saturated triacylglycerols were chosen according to a four-factor central composite design. The four factors are the length of the main chain (X_1), the branching position (X_2), the length of the side chain (X_3), and the number of branched fatty acyl groups attached to the glycerol (X_4). As can be seen in Table 1, the different levels of variable X_2 are defined as functions of variable X_1. In a similar way, the levels of variable X_3 are defined as functions of variables X_1 and X_2. This is due to the fact that, in the case of the branching position, level +1 represents a carbon atom near the end of the main chain. Depending on the length of the main chain, the number of the carbon atom to which the branch is affixed will vary. In the same way, the length of the branch has an upper limit. If this limit is passed, the branch has become the main chain. In the case of the branching position, level +1 has been chosen as a position two carbons away from the end of the main chain to be able to vary the length of the branch.

TABLE 1
Factor Levels of the Experimental Design

	Level		
Factor	–1	0	+1
Length of main chain (X_1)	6	12	18
Branching position (X_2)	2	$X_1/2$	$X_1 - 2$
Length of side chain (X_3)	0	$(X_1 - X_2 + 1)/2$	$X_1 - X_2$
Number of branched acyl groups (X_4)	1	2	3

The complete experimental design, including four control substances, is shown in Table 2. As can be seen, the axial points have been placed 1 unit away from the center instead of the ideal distance of 2 units. This variant of the central composite design is called central composite face-centered design.

The straight chain acid (which is present when there are fewer than three branched fatty acids present in the triacylglycerol) has been chosen to be dodecanoic acid for the axial and center points and hexanoic or octadecanoic acid (half of each) for the remaining triacylglycerols (this is also the way the straight chain acid would have been chosen if the length of the straight chain had been treated as a fifth variable in a fractional factorial design). This way of designing the experiment is based on the idea that the branched fatty acid to a great extent determines the behavior of the entire triacylglycerol.

Statistical analyses. The evaluation of the results was made with the help of the software MODDE for Windows, version 3.0 (Umetri AB, Umeå, Sweden). All measurements were done in a randomized order. Measurements in the center point of the design were repeated twice. MODDE uses multiple linear regression to calculate a prediction equation for each response:

$$Y_p = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 \quad [1]$$

The estimated coefficients are then rejected or retained depending on their probability. The test is performed at a significance level of $\alpha = 0.05$. The significant coefficients are then reestimated to give the final prediction equation. Outliers, identified by normal probability plots of the residuals (14), are excluded from the multiple linear regression. The fact that a compound has been identified as an outlier means that it does not fit into the mathematical description of a particular physical property. It does not mean that the measurements are incorrect or that the wrong substance has been synthesized. The subject has been described in a recent review article (15).

RESULTS AND DISCUSSION

Gel point. The coefficients of the final prediction equation of the gel point are shown in Table 3 with the corresponding probabilities and 95% confidence intervals. Four triacylglycerols have been identified as outliers (by the technique of normal probability plots) and are consequently excluded from the regression calculations. The four compounds are the triacylglycerols with hexanoyl groups in two positions and a branched fatty acyl group in the remaining position (all marked in Table 4). The two control triacylglycerols with deviating gel points both have two stearoyl groups attached to the glycerol. This could explain why their gel points are high compared to their predicted values (in contrast to the other

TABLE 2
Experimental Design Including Four Control Substances

Branched triacylglycerol	Length of main chain (X_1)	Branching position (X_2)	Length of side chain (X_3)	Number of branched acyl groups (X_4)
Triacylglycerols included in the design				
1-(2-Methylhexanoyl)-2,3-dioctadecanoyl-glycerol	−1	−1	−1	−1
1,2-Dihexanoyl-3-(2-methyloctadecanoyl)-glycerol	+1	−1	−1	−1
1,2-Dihexanoyl-3-(4-methylhexanoyl)-glycerol	−1	+1	−1	−1
1-(16-Methyloctadecanoyl)-2,3-dioctadecanoyl-glycerol	+1	+1	−1	−1
1-(2-Butylhexanoyl)-2,3-dihexanoyl-glycerol	−1	−1	+1	−1
1-(2-Hexadecyloctadecanoyl)-2,3-dioctadecanoyl-glycerol	+1	−1	+1	−1
1-(4-Ethylhexanoyl)-2,3-dioctadecanoyl-glycerol	−1	+1	+1	−1
1-(16-Ethyloctadecanoyl)-2,3-dihexanoyl-glycerol	+1	+1	+1	−1
Tris(2-methylhexanoyl)-glycerol	−1	−1	−1	+1
Tris(2-methyloctadecanoyl)-glycerol	+1	−1	−1	+1
Tris(4-methylhexanoyl)-glycerol	−1	+1	−1	+1
Tris(16-methyloctadecanoyl)-glycerol	+1	+1	−1	+1
Tris(2-butylhexanoyl)-glycerol	−1	−1	+1	+1
Tris(2-hexadecyloctadecanoyl)-glycerol[a]	+1	−1	+1	+1
Tris(4-ethylhexanoyl)-glycerol	−1	+1	+1	+1
Tris(16-ethyloctadecanoyl)-glycerol	+1	+1	+1	+1
1-Dodecanoyl-2,3-bis(6-propyldodecanoyl)-glycerol	0	0	−0.2	0
1-Dodecanoyl-2,3-bis(3-ethylhexanoyl)-glycerol	−1	0	0	0
1-Dodecanoyl-2,3-bis(9-pentyloctadecanoyl)-glycerol	+1	0	0	0
1-Dodecanoyl-2,3-bis(2-pentyldodecanoyl)-glycerol	0	−1	−0.111	0
1-Dodecanoyl-2,3-bis(10-methyldodecanoyl)-glycerol	0	+1	−1	0
1-Dodecanoyl-2,3-bis(6-methyldodecanoyl)-glycerol	0	0	−1	0
1-Dodecanoyl-2,3-bis(6-hexyldodecanoyl)-glycerol	0	0	+1	0
1,2-Didodecanoyl-3-(6-propyldodecanoyl)-glycerol	0	0	−0.2	−1
Tris(6-propyldodecanoyl)-glycerol	0	0	−0.2	+1
Control triacylglycerols				
1-Hexanoyl-2,3-bis(6-propyldodecanoyl)-glycerol	0	0	−0.2	0
1-Octadecanoyl-2,3-bis(6-propyldodecanoyl)-glycerol	0	0	−0.2	0
1,2-Didodecanoyl-3-(16-methyloctadecanoyl)-glycerol	+1	+1	−1	−1
1,2-Dioctadecanoyl-3-(6-propyldodecanoyl)-glycerol	0	0	−0.2	−1

[a]This compound was not synthesized in sufficient amounts to perform measurements.

outliers which have short main chains and correspondingly low gel points). The resulting equation is:

$$Y_{p,\text{ gel point}} = -57 + 23\,X_1 - 1.0\,X_2 - 30\,X_4 + 20\,X_1X_4 + 48\,X_2^2 + 16\,X_4^2 \quad [2]$$

The length of the side chain (X_3) is the only factor which does not have an influence on the response. Figure 1 shows a contour plot of the response surface for triacylglycerols having three branched fatty acyl groups attached to the glycerol skeleton. The response surfaces for the other types of triacylglycerols (having one or two branched fatty acids) have the same appearance. It can be seen that the lowest gel points are obtained when the branch is situated on the middle of the main chain. This result is analogous to a previous investigation on the branched fatty acids themselves (16).

The observed and the predicted gel points are compared in Table 4. An analysis of variance (ANOVA) scheme is shown in Table 5. The coefficient of multiple determination is R^2 = 0.961.

Refractive index (60°C). Four compounds have been identified as outliers, three of which are the same as for the gel point prediction equation. All outliers are marked in Table 4. The calculated response function (the parameters of which

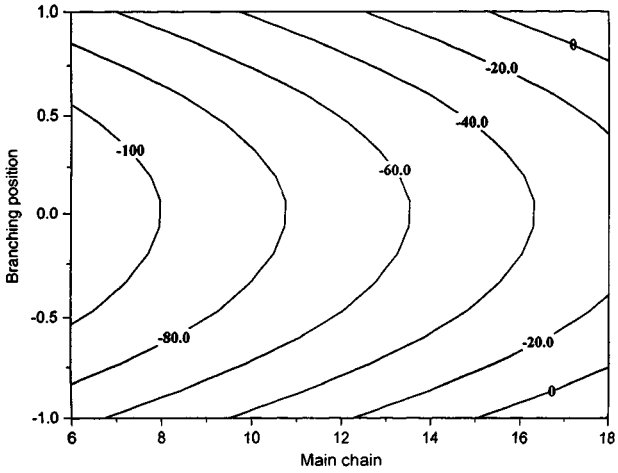

FIG. 1. Contour plot showing the gel point as a function of the structure for monoalkyl branched saturated homotriacylglycerols. The length of the main chain (X_1) is given as the number of carbons (6 to 18) while the branching position (X_2) is given as coded values (−1 to +1).

TABLE 3
Coefficients from the Final Prediction Equations of the Gel Point, the Refractive Index, and the Density

Term	Coefficient[a]	Probability[b]
Gel point		
Constant	−57 ± 8	0.00
Main chain (X_1)	23 ± 6	0.00
Branching position (X_2)	−1.0 ± 6	0.75
Number of branches (X_4)	−30 ± 6	0.00
Main chain × number of branches (X_1X_4)	20 ± 7	0.00
(branching position)^2 (X_2^2)	48 ± 12	0.00
(number of branches)^2 (X_4^2)	16 ± 12	0.01
Refractive index		
Constant	1.4432 ± 0.0008	0.00
Main chain (X_1)	0.0046 ± 0.0010	0.00
Branching position (X_2)	0.0003 ± 0.0010	0.50
Side chain (X_3)	0.0010 ± 0.0010	0.06
Number of branches (X_4)	−0.0018 ± 0.0010	0.00
Main chain × branching position (X_1X_2)	−0.0018 ± 0.0011	0.01
Main chain × number of branches (X_1X_4)	0.0038 ± 0.0012	0.00
Branching position × side chain (X_2X_3)	-0.0014 ± 0.0011	0.02
Side chain × number of branches (X_3X_4)	0.0025 ± 0.0011	0.00
Density		
Constant	0.8906 ± 0.0037	0.00
Main chain (X_1)	−0.0145 ± 0.0047	0.00
Branching position (X_2)	0.0108 ± 0.0050	0.00
Side chain (X_3)	−0.0090 ± 0.0051	0.00
Number of branches (X_4)	0.0012 ± 0.0049	0.62
Main chain × number of branches (X_1X_4)	−0.0189 ± 0.0053	0.00

[a]Values are given with 95% confidence intervals. [b]The probability that a coefficient is equal to zero.

can also be found in Table 3 together with 95% confidence intervals and values of probability) is:

$$Y_{p,\text{ refractive index (60°C)}} = 1.4432 + 0.0046\ X_1 + 0.0003\ X_2 + 0.0010\ X_3 - 0.0018\ X_4 - 0.0018\ X_1X_2 + 0.0038\ X_1X_4 - 0.0014\ X_2X_3 + 0.0025\ X_3X_4 \quad [3]$$

All four factors are important to the response, which means that two factors must be set to a fixed value in order to make a contour plot possible. Since the branching position is the least important factor, it has been set to $X_2 = 0$, i.e., a branching in the middle of the main chain. In this way it is possible to visualize the response function (Fig. 2). Also in this case the homotriacylglycerols have been chosen for the figure. One conclusion is that the longer the main chain the higher the refractive index. This is also similar to the properties of the branched fatty acids themselves (16). The observed and the predicted values are compared in Table 4. An ANOVA scheme is shown in Table 5. The coefficient of multiple determination is $R^2 = 0.950$.

Density (60°C). In addition to the three compounds for which the density could not be measured, two more triacylglycerols were excluded from the regression calculations (Table 4). The obtained prediction equation is:

$$Y_{p,\text{ density (60°C)}} = 0.8906 - 0.0145\ X_1 + 0.0108\ X_2 - 0.0090\ X_3 + 0.0012\ X_4 - 0.0189\ X_1X_4 \quad [4]$$

As in the refractive index case, it is not possible to show the response function in a single picture. All four factors are important. Just to give the reader some idea of the appearance of the response surfaces, one of them is shown in Figure 3. This picture of the homotriacylglycerols demonstrates the decreasing effect of a longer side chain and the increasing ef-

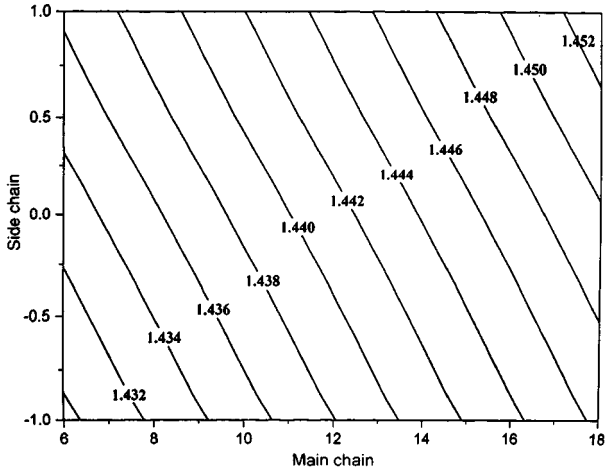

FIG. 2. Contour plot showing the refractive index (at 60°C) as a function of the structure for monoalkyl branched saturated homotriacylglycerols. The length of the main chain (X_1) is given as the number of carbons (6 to 18), and the length of the side chain (X_3) is given as coded values (−1 to +1). In this plot the branching position is set to a medium position ($X_2 = 0$).

TABLE 4
Gel Points, Refractive Indices, and Densities of Monoalkyl Branched Triacylglycerols

Branched triacylglycerol	Gel point			Refractive index (60°C)			Density (60°C)		
	Observed (°C)	Predicted[a] (°C)	Residual (°C)	Observed	Predicted[a]	Residual	Observed	Predicted[a]	Residual
Triacylglycerols included in the design									
1-(2-Methylhexanoyl)-2,3-dioctadecanoyl-glycerol	32	34 ± 16	2	1.4429	1.4422 ± 0.0028	0.0007	0.8841	0.8833 ± 0.0114	0.0008
1,2-Dihexanoyl-3-(2-methyloctadecanoyl)-glycerol[b,c]	–16	40 ± 15	56	1.4350	1.4474 ± 0.0043	–0.0124	0.8989	0.8921 ± 0.0108	0.0068
1,2-Dihexanoyl-3-(4-methylhexanoyl)-glycerol[b,c,d]	–71	32 ± 16	103	1.4300	1.4493 ± 0.0035	0.0193	0.9458	0.9048 ± 0.0126	0.0410
1-(16-Methyloctadecanoyl)-2,3-dioctadecanoyl-glycerol	43	38 ± 16	5	1.4490	1.4473 ± 0.0027	0.0017	—[e]	0.9136 ± 0.0136	—
1-(2-Butylhexanoyl)-2,3-dihexanoyl-glycerol[b,c,d]	–67	34 ± 16	101	1.4305	1.4421 ± 0.0044	–0.0116	0.9280	0.8654 ± 0.0129	0.0626
1-(2-Hexadecyloctadecanoyl)-2,3-dioctadecanoyl-glycerol	50	40 ± 16	10	1.4485	1.4472 ± 0.0028	0.0013	0.8642	0.8742 ± 0.0101	–0.0100
1-(4-Ethylhexanoyl)-2,3-dioctadecanoyl-glycerol	32	32 ± 16	±0	1.4442	1.4434 ± 0.0029	0.0008	0.8865	0.8869 ± 0.0116	-0.0004
1-(16-Ethyloctadecanoyl)-2,3-dihexanoyl-glycerol[b]	–41	38 ± 16	–79	1.4395	1.4414 ± 0.0030	–0.0019	0.9053	0.8957 ± 0.0106	0.0096
Tris(2-methylhexanoyl)-glycerol	–70	–65 ± 12	–5	1.4271	1.4260 ± 0.0029	0.0012	0.9269	0.9234 ± 0.0096	0.0035
Tris(2-methyloctadecanoyl)-glycerol	28	21 ± 15	7	1.4452	1.4464 ± 0.0028	–0.0012	0.8704	0.8566 ± 0.0109	0.0138
Tris(4-methylhexanoyl)-glycerol	–70	–67 ± 12	–3	1.4325	1.4330 ± 0.0025	–0.0005	0.9390	0.9449 ± 0.0112	–0.0059
Tris(16-methyloctadecanoyl)-glycerol	30	19 ± 13	11	1.4478	1.4463 ± 0.0028	0.0015	0.8749	0.8781 ± 0.0111	–0.0032
Tris(2-butylhexanoyl)-glycerol	–58	–65 ± 12	7	1.4343	1.4357 ± 0.0029	–0.0014	0.9026	0.9055 ± 0.0108	–0.0029
Tris(4-ethylhexanoyl)-glycerol	–76	–67 ± 12	–9	1.4380	1.4370 ± 0.0029	0.0010	0.9367	0.9270 ± 0.0096	0.0097
Tris(16-ethyloctadecanoyl)-glycerol	10	19 ± 13	–9	1.4499	1.4503 ± 0.0027	–0.0004	—[e]	0.8602 ± 0.0136	—
1-Dodecanoyl-2,3-bis(6-propyldodecanoyl)-glycerol	–56	-57 ± 8	1	1.4431	1.4430 ± 0.0008	0.0001	0.8884	0.8924 ± 0.0036	–0.0040
	–55	–57 ± 8	2	1.4431	1.4430 ± 0.0008	0.0001	0.8885	0.8924 ± 0.0036	–0.0039
	–58	–57 ± 8	–1	1.4431	1.4430 ± 0.0008	0.0001	0.8883	0.8924 ± 0.0036	–0.0041
1-Dodecanoyl-2,3-bis(3-ethylhexanoyl)-glycerol	–64	–81 ± 10	17	1.4372	1.4386 ± 0.0013	–0.0014	0.9152	0.9051 ± 0.0057	0.0101
1-Dodecanoyl-2,3-bis(9-pentyloctadecanoyl)-glycerol	–51	–34 ± 10	–17	1.4471	1.4478 ± 0.0013	–0.0007	0.8741	0.8761 ± 0.0061	–0.0020
1-Dodecanoyl-2,3-bis(2-pentyldodecanoyl)-glycerol	–25	-8 ± 14	–17	1.4420	1.4426 ± 0.0013	–0.0006	0.8786	0.8809 ± 0.0058	–0.0023
1-Dodecanoyl-2,3-bis(10-methyldodecanoyl)-glycerol	–4	–10 ± 13	6	1.4416	1.4440 ± 0.0017	–0.0024	—[e]	0.9103 ± 0.0087	—
1-Dodecanoyl-2,3-bis(6-methyldodecanoyl)-glycerol	–52	–57 ± 8	5	1.4410	1.4422 ± 0.0012	–0.0012	0.8935	0.8996 ± 0.0058	–0.0061
1-Dodecanoyl-2,3-bis(6-hexyldodecanoyl)-glycerol	–55	-57 ± 8	2	1.4448	1.4441 ± 0.0013	0.0007	0.8809	0.8817 ± 0.0067	–0.0008
1,2-Didodecanoyl-3-(6-propyldodecanoyl)-glycerol[c]	–24	–12 ± 14	–12	1.4420	1.4453 ± 0.0015	–0.0033	0.8910	0.8913 ± 0.0063	–0.0003
Tris(6-propyldodecanoyl)-glycerol	–70	–71 ± 13	1	1.4431	1.4407 ± 0.0011	0.0024	0.8851	0.8936 ± 0.0058	–0.0085
Control triacylglycerols									
1-Hexanoyl-2,3-bis-(6-propyldodecanoyl)-glycerol	–60	–57 ± 8	–3	1.4410	1.4430 ± 0.0008	–0.0020	0.8979	0.8924 ± 0.0036	0.0055
1-Octadecanoyl-2,3-bis-(6-propyldodecanoyl)-glycerol	–19	–57 ± 8	38	1.4450	1.4430 ± 0.0008	0.0020	0.8816	0.8924 ± 0.0036	–0.0108
1,2-Didodecanoyl-3-(16-methyloctadecanoyl)-glycerol	25	38 ± 16	–13	1.4390	1.4473 ± 0.0027	–0.0083	0.8686	0.9136 ± 0.0136	–0.0450
1,2-Dioctadecanoyl-3-(6-propyldodecanoyl)-glycerol	29	–12 ± 14	41	1.4459	1.4453 ± 0.0015	0.0006	0.8781	0.8913 ± 0.0063	–0.0132

[a]Values are given together with their 95% confidence intervals. [b]Outlier in the gel point evaluation. [c]Outlier in the refractive index evaluation. [d]Outlier in the density evaluation. [e]Insufficient material to perform the measurements.

TABLE 5
Analysis of Variance Scheme and Residual Sum of Squares Breakdown for the Three Prediction Equations

	Gel point			Refractive index			Density		
Source of variation	Sum of squares	Degrees of freedom	Mean square	Sum of squares	Degrees of freedom	Mean square	Sum of squares	Degrees of freedom	Mean square
Constant	14 408	1	14 408	45.7489	1	45.7489	16.7823	1	16.7823
Model terms	39 190	6	6 532	6.38×10^{-4}	8	7.97×10^{-5}	0.0077	5	0.0015
Residuals	1 592	15	106	3.39×10^{-5}	13	2.61×10^{-6}	0.0009	15	0.0001
Total	55 189	22	2 509	45.7496	22	2.0795	16.7909	21	0.7996
Residual sum of squares breakdown									
Pure error	4.7	2	2.3	0	2	0	8.65×10^{-8}	2	4.33×10^{-8}
Lack of fit	1 587	13	122	3.39×10^{-5}	11	3.08×10^{-6}	8.59×10^{-4}	13	6.61×10^{-5}

fect of a branch far out on the main chain. A longer main chain gives a higher density only when there are two or three branched fatty acyl groups attached to the glycerol. The ANOVA scheme is shown in Table 5. The coefficient of multiple determination is $R^2 = 0.900$. The density prediction equation is the least accurate of the three prediction equations.

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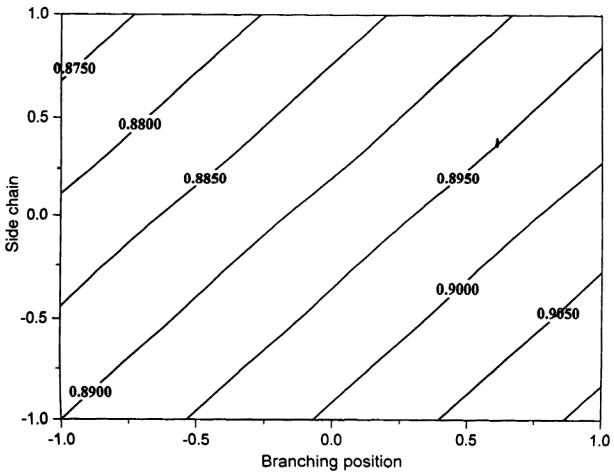

FIG. 3. Contour plot showing the density (at 60°C) as a function of the structure for monoalkyl branched saturated homotriacylglycerols. Both the branching position (X_2) and the length of the side chain (X_3) are given as coded values (–1 to +1). The length of the main chain (X_1) is fixed at a medium position ($X_1 = 12$).

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On the Syntheses of Triacylglycerols from Branched Saturated Fatty Acids

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ABSTRACT: A number of triacylglycerols with branched acyl groups were prepared *via* 1,2-isopropylidene glycerol for the purpose of studying three different physical properties: gel point, refractive index, and density. The monoacid triacylglycerols were prepared either *via* the corresponding acids or the acyl chlorides.
Lipids 32, 667–673 (1997).

Monoalkyl branched saturated triacylglycerols possess physical properties very different from triacylglycerols with straight-chain acyl groups (1,2). Aydin *et al.* (1) have synthesized both the 1-monoacylglycerols and the monoacid triacylglycerols of nine different α-monoalkyl branched fatty acids and measured several different physical properties of these compounds.

In order to investigate the relation between the structure of the monoalkyl branched saturated triacylglycerols and their physical properties, a four-factor central composite design was constructed. The four factors are the length of the main chain, the length of the side chain, the branching position, and the number of branched acyl groups attached to the glycerol backbone. The first three structure variables are related to branched acyl groups, whereas the fourth variable defines the triacylglycerol. This design requires 25 different triacylglycerols to be prepared. The result of this investigation is given in the accompanying paper (3).

MATERIALS AND METHODS

^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Varian XL-300 spectrometer (Palo Alto, CA). In the interpretation of ^{1}H NMR spectra, *s* is used for singlet, *d* for doublet, *t* for triplet, *qu* for quartet, *qv* for quintet, *sex,* for sextet, and *oct* for octuplet. The infrared spectrometer used was a Perkin Elmer Model 298 (Norwalk, CT). Mass spectra were recorded on a Finnigan 4021 (San Jose, CA) and a Jeol JMS-Sx 1200 spectrometer (Tokyo, Japan). Gas chromatograms were obtained by using Varian gas chromatograph models 1400 and 3300. Glass columns (length 2.0 m, i.d. 2 mm) were used with 10% SP-2340 on Chromosorb 100/120 and 3% OV 101 on Gas. Chrom. Q 100/120. High-performance liquid chromatography (HPLC) separations were performed by using equipment containing Gilson pump 305 (Gilson Medical Electronics, Middleton, WI), a Dynamex RP C 18 preparative column (250 × 2.0 cm) (Rainin Instrument Co., Woburn, MA), and a Gilson IR detector 131. Reactions which needed to be performed under anhydrous conditions were run in dry vessels under nitrogen. All acid chlorides were prepared from the corresponding acids (4) and an excess of oxalyl chloride (5). Pyridine was dried and purified by distillation from barium oxide. Glycerol was freshly distilled, dichloromethane was distilled from phosphorus pentoxide, methoxymethanol was distilled from molecular sieves (4 Å), and toluene was distilled from sodium wire and benzophenone.

General procedure for the preparation of 1-acyl-2,3-isopropylidene glycerol (Procedure A). A three-necked flask equipped with condenser with drying tube, dropping funnel, and magnetic bar was surrounded with an ice-water bath and flushed with nitrogen. Isopropylidene glycerol (1.25 equiv.) and pyridine (1.25 equiv.) were dissolved in toluene (4.30 mL/mmol of acid chloride). At 0°C, the acid chloride (1.00 equiv.) was added dropwise, and when the addition was complete the cooling bath was removed and the reaction mixture was stirred under nitrogen at room temperature for 20 h. The solution was washed with water, sodium carbonate solution, and water; dried over magnesium sulfate; evaporated; and chromatographed on neutral alumina (100 g/0.1 mol of acylisopropylidene glycerol) using toluene as the eluant. Toluene was evaporated and the residue dried *in vacuo* (2 mm Hg) at 60°C for 4–5 h.

General procedure for the preparation of triacylglycerols (Procedure B). A three-necked flask equipped with reflux condenser with drying tube and magnetic bar was flushed

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Abbreviations: EI, electron ionization; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; NMR, nuclear magnetic resonance.

with nitrogen, after which the flask was charged with methoxymethanol (3.0 mL/mmol of acylisopropylidene glycerol). Under stirring, boric acid (16 equiv.) was added and the mixture heated to 100°C and stirred for about 3 h. By thin-layer chromatography, it was confirmed that the starting material was consumed and by GC that no isomerization had occurred. After cooling, the reaction mixture was poured onto ice and water, and ice-cooled dichloromethane was added. Upon shaking vigorously, the boric acid precipitated and was filtered off, and the precipitate was washed twice with ice-cooled dichloromethane. The combined organic phases were washed with ice-cooled water, dried with sodium sulfate under magnetic stirring for 2 h, and evaporated at room temperature. The residue was dissolved in dichloromethane (3.7 mL/mmol of 1-acylglycerol) and cooled to 0°C. Under stirring, pyridine was added, whereupon the appropriate acyl chloride was added dropwise. After removing the cooling bath, the reaction mixture was stirred under nitrogen for 72 h and then poured into water. The phases were separated and the organic phase washed with water, dried over magnesium sulfate, evaporated, and flash chromatographed on silica gel.

General procedure to the preparation of monoacid triacylglycerols (Procedure C). The acid (169 mmol), glycerol (47 mmol), and 4-toluenesulfonic acid (3 mmol) were dissolved in toluene (40 mL). The reaction mixture was stirred at 180°C for 40 h. After cooling, diethyl ether was added (20 mL), and the solution was washed twice with saturated sodium carbonate solution and twice with water whereupon it was dried over magnesium sulfate. The crude product was purified by column chromatography on neutral alumina.

(Procedure D). A dried, nitrogen-filled flask was charged with glycerol (1.00 g, 10.7 mmol) and pyridine (3.90 mL, 48.1 mmol) in dichloromethane (80 mL), and the solution was cooled to 0°C. Under stirring, 16-methyloctadecanoyl chloride (15.2 g, 48.1 mmol) was added. After removing the ice bath, the reaction mixture was stirred at room temperature for 3 d and water (75 mL) was added. The phases were separated and the organic phase washed twice with water and dried over magnesium sulfate. The crude product was flash chromatographed on silica.

1-Hexanoyl-2,3-isopropylidene glycerol (**1**) (6,7) was prepared according to procedure A from 1,2-isopropylidene glycerol (6.60 g, 50.0 mmol), pyridine (3.95 g, 50.0 mmol), toluene (172 mL), and hexanoyl chloride (5.06 g, 40.0 mmol). Evaporation and distillation (72–78°C/0.3 mm Hg) gave **1** (6.30 g, 71%).

1-(2-Methylhexanoyl)-2,3-isopropylidene glycerol (**2**) was prepared according to procedure A from 1,2-isopropylidene glycerol (6.35 g, 48.1 mmol), pyridine (3.87 mL, 48.1 mmol) and 2-methylhexanoyl chloride (5.70 g, 38.5 mmol) in dry toluene (140 mL). After workup, **2** (5.0 g, 53%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 3H, CH_3)=, 1.15 (*d*, 3H, CH_3), 1.29 (*m*, 4H, CH_2), 1.36 (*s*, 3H, iso-CH_3), 1.43 (*s*, 3H, iso-CH_3), 1.60 (*m*, 2H, CH_2), 2.47 (*m*, 2H, CH_2), 3.74 (*oct*, 1H), 4.10 (*m*, 3H), 4.30 (*m*, 1H). Mass spectrometry (MS) electron ionization (EI) *m/e* (rel. int.) 229 (88) M − 15, 113 (40), 101 (100), 85 (73), 69 (50), 57 (38), 43 (60).

1-(4-Methylhexanoyl)-2,3-isopropylidene glycerol (**3**) was prepared according to procedure A from 1,2-isopropylidene glycerol (8.87 g, 67.2 mmol), pyridine (5.41 mL, 67.2 mmol), and 4-methylhexanoyl chloride in dry toluene (190 mL). After workup, **3** (77.9 g, 60%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.87 (*t*, 3H, CH_3), 0.86 (*d*, 3H, CH_3), 1.37 (*s*, 3H, iso-CH_3), 1.40 (*m*, 5H, CH_2, C*H*), 1.44 (*s*, 3H, iso-CH_3), 2.35 (*m*, 2H, CH_2), 3.74 (*qu*, 1H), 4.12 (*m*, 3H), 4.32 (*oct*, 1H). MS (EI) *m/e* (rel. int.) 229 (6) M − 15, 113 (8), 101 (17), 95 (13), 57(23), 43 (100).

1-(4-Ethylhexanoyl)-2,3-isopropylidene glycerol (**4**) was prepared according to procedure A from 1,2-isopropylidene glycerol (2.6 g, 19.4 mmol), pyridine (1.52 mL, 19.0 mmol), and 4-ethylhexanoyl chloride (2.3 g, 14.4 mmol) in dry toluene (100 mL). After workup, **4** (3.4 g, 94%) was obtained.

1-(2-Butylhexanoyl)-2,3-isopropylidene glycerol (**5**) was prepared according to procedure A from 1,2-isopropylidene glycerol (7.67 g, 58.1 mmol), pyridine (4.68 mL, 58.1 mmol), and 2-butylhexanoyl chloride (8.83 g, 46.5 mmol). After workup, **5** (9.8 g, 66%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 6H, CH_3), 1.26 (*m*, 8H, CH_2), 1.37 (*s*, 3H, iso-CH_3), 1.43 (*s*, 3H, iso-CH_3), 1.46 (*m*, 2H, CH_2), 1.59 (*m*, 2H, CH_2), 2.40 (*m*, 1H, C*H*), 3.74 (*qu*, 1H), 4.07 (*qu*, 1H), 4.14 (*d*, 2H), 3.41 (*qv*, 1H). MS (EI) *m/e* (rel. int.) 271 (7) M − 15, 101 (22), 71 (28), 57 (42), 43 (100).

1-Dodecanoyl-2,3-isopropylidene glycerol (**6**) (7) was prepared according to procedure A from 1,2-isopropylidene glycerol (4.2 g, 31 mmol), pyridine (2.5 mL, 31.0 mmol), and dodecanoyl chloride (5.45 g, 25.0 mmol) in dry toluene (125 mL). After workup, **6** (4.7 g, 60%) was obtained.

1-(6-Propyldodecanoyl)-2,3-isopropylidene glycerol (**7**) was prepared according to procedure A from 1,2-isopropylidene glycerol (3.30 g, 25.0 mmol), pyridine (1.98 g, 25.0 mmol), toluene (86 mL), and 6-propyldodecanoyl chloride (5.21 g, 20.0 mmol). Evaporation and drying gave **7** (4.63 g, 65.0%). ^{1}H NMR ($CDCl_3$) δ 0.88 (*m*, 6H, CH_3), 1.25 (*m*, 19H, CH_2, C*H*), 1.37 (*s*, 3H, iso-CH_3), 1.44 (*s*, 3H, iso-CH_3), 1.61 (*m*, 2H, CH_2), 2.35 (*t*, 2H, CH_2), 3.74 (*qu*, 1H), 4.10 (*m*, 3H), 4.32 (*m*, 1H). MS (EI) *m/e* (rel. int.) 341 (100) M − 15, 299 (15), 255 (11), 213 (11), 180 (23), 129 (44), 101 (51), 57 (24), 43 (18).

1-Octanoyl-2,3-isopropylidene-glycerol (**8**) (7) was prepared according to procedure A from 1,2-isopropylidene glycerol (5.29 g, 40.1 mmol), pyridine (3.17 mL, 40.1 mmol), toluene (160 mL), and octadecanoyl chloride (9.62 g, 32.0 mmol). Evaporation and drying gave **8** (7.64 g, 60%).

1-(2-Methyloctadecanoyl)-2,3-isopropylidene glycerol (**9**) was prepared according to procedure A from isopropylidene glycerol (5.5 g, 42.0 mmol), pyridine (3.4 mL, 42.0 mmol), and 2-methyloctadecanoyl chloride (10.6 g, 33.6 mmol) in dry toluene (168 mL). After workup, **9** (10.7 g, 77%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 3H, CH_3), 1.15 (*d*, 3H, CH_3), 1.25 (*s*, 28H, CH_2), 1.37 (*s*, 3H, iso-CH_3), 1.44 (*s*, 3H, iso-CH_3), 1.65 (*m*, 2H, CH_2), 2.47 (*sex*, 1H, C*H*), 3.70 (*oct*,

1H), 4.10 (*m*, 3H), 4.30 (*qv*, 1H). MS (EI) *m/e* (rel. int.) 397 (7) M – 15, 185 (6), 143 (8), 57 (47), 43 (100).

1-(16-Methyloctadecanoyl)-2,3-isopropylidene glycerol (**10**) was prepared according to procedure A from 1,2-isopropylidene glycerol (0.88 g, 6.63 mmol), pyridine (0.54 mL, 6.63 mmol), toluene (23 mL), and 16-methyloctadecanoyl chloride (1.68 g, 5.30 mmol). Evaporation and drying gave **10** (1.70 g, 76.0%).

1-(16-Ethyloctadecanoyl)-2,3-isopropylidene-glycerol (**11**) was prepared according to procedure A from 1,2-isopropylidene glycerol (5.29 g, 40.1 mmol), pyridine (3.17 mL, 40.1 mmol), and 16-ethylhexanoyl chloride (10.6 g, 32.0 mmol) in dry toluene (160 mL). After workup, **11** (8.3 g, 61%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.83 (*t*, 6H, CH_3), 1.25 (*m*, 33H, CH_2, CH), 1.37 (*s*, 3H, iso-CH_3), 1.44 (*s*, 3H, iso-CH_3), 1.62 (*m*, 2H, CH_2), 2.34 (*t*, 2H, CH_2), 3.74 (*qu*, 1H), 4.12 (*m*, 3H), 4.32 (*oct*, 1H). MS (EI) *m/e* (rel. int.) 411 (5) M – 15, 185 (3), 171 (5), 129 (10), 101 (27), 57 (35), 43 (100).

1-(2-Hexadecyloctadecanoyl)-2,3-isopropylidene glycerol (**12**) was prepared according to procedure A from 1,2-isopropylidene glycerol (3.25 g, 24.6 mmol), pyridine (1.98 mL, 24.6 mmol), and 2-hexadecyloctadecanoyl chloride (10.3 g, 19.7 mmol) in toluene (72 mL). After workup toluene as the eluent was changed for pentane/ether (88:12), and **12** (7.8 g, 64%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.80 (*t*, 6H, CH_3), 1.25 (*m*, 56H, CH_2), 1.37 (*s*, 3H, iso-CH_3), 1.43 (*s*, 3H, CH_3), 2.37 (*m*, 1H, C*H*), 3.70 (*qu*, 1H), 4.07 (*qu*, 2H), 4.13 (*d*, 2H), 4.31 (*qv*, 1H). MS (EI) *m/e* (rel. int.) 607 (82) M – 15, 564 (7), 340 (10), 137 (20), 97 (37), 81 (47), 69 (100), 57 (58), 43 (42).

1-(4-Methylhexanoyl)-2,3-dihexanoyl glycerol (**13**) was prepared from procedure B from 1-(4-methylhexanoyl)-2,3-isopropylidene glycerol (**3**) (7.80 g, 32.0 mmol), pyridine (12.3 g, 153 mmol), and hexanoyl chloride (20.6 g, 153 mmol). After flash chromatography (ethyl acetate/petroleum ether, 1:9) and HPLC (acetonitrile) **13** (4.30 g, 34%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*m*, 12H, CH_3), 1.31 (*m*, 10H, CH_2), 1.62 (*m*, 6H, CH_2), 2.32 (*m*, 7H, CH_2, C*H*), 4.15 (*qu*, 2H), 4.30 (*qu*, 2H), 5.27 (*m*, 1H). MS (EI) *m/e* (rel. int.) 285 (12) M – 115, 271 (24), 113 (38), 99 (100), 71 (23), 43 (24). Calc. for $C_{22}H_{40}O_6$: C 65.96; H 10.07; MW 400.54. Found: C 66.7; H 10.2.

1-(2-Butylhexanoyl)-2,3-dihexanoyl glycerol (**14**) was prepared according to procedure B from 1-(2-butylhexanoyl)-2,3-isopropylidene glycerol (**5**) (8.0 g, 28.0 mmol), pyridine (5.55 mL, 68.8 mmol), and hexanoyl chloride (18.0 g, 134 mmol). After flash chromatography (ethyl acetate/petroleum ether, 7.5:92.5) and HPLC (acetonitrile), **14** (7.2 g, 60%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.89 (*m*, 12H, CH_3), 1.29 (*m*, 18H, CH_2), 1.62 (*m*, 8H, CH_2), 2.31 (*m*, 5H, CH_2, C*H*), 4.14 (*oct*, 2H), 4.32 (*oct*, 2H), 5.27 (*m*, 1H). MS (EI) *m/e* (rel. int.) 385 +1 (17) M – 57, 327 (70), 271 (77), 155 (50), 99 (100), 71 (42), 43 (39). Calc. for $C_{25}H_{46}O_6$: C 67.83; H 10.48; MW 442.63. Found C 68.3; H 10.4.

1-Dodecanoyl-2,3-di-3-ethylhexanoyl glycerol (**15**) was obtained according to procedure B from 1-dodecanoyl-2,3-isopropylidene glycerol (**6**) (5.10 g, 16.2 mmol), pyridine (3.21 mL, 39.8 mmol), and 3-ethylhexanoyl chloride (6.30 g, 39.8 mmol). After flash chromatography (ethyl acetate/pentane, 8:92) and HPLC (acetonitrile/ethyl acetate, 85:15), **15** (6.07 g, 71%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*m*, 15H, CH_3), 1.30 (*m*, 28H, CH_2), 1.60 (*m*, 2H, CH_2), 1.81 (*m*, 2H, C*H*), 2.31 (*t*, 2H, CH_2), 2.25 (*qu*, 4H, CH_2), 4.14 (*oct*, 2H), 4.30 (*oct*, 2H), 5.27 (*m*, 1H). Calc. for $C_{31}H_{58}O_6$: C 70.68; H 11.10; MW 526.77. Found: C 70.7; H 10.8; $(M + NH_4)^+$ 544.

1-(2-Methyloctanoyl)-2,3-dihexanoyl glycerol (**16**) was prepared according to procedure B from 1-(2-methyloctadecanoyl)-2,3-isopropylidene glycerol (**9**) (10.7 g, 26 mmol), pyridine (10.1 mL, 125 mmol), and hexanoyl chloride (17.3 mL, 125 mmol). After flash chromatography (ethyl acetate/petroleum ether, 1:9) and HPLC (chloroform/methanol, 20:80), **16** (4.40 g, 26%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.89 (*m*, 9H, CH_3), 1.13 (*d*, 3H, CH_3), 1.26 (*s*, 36H, CH_2), 1.61 (*m*, 6H, CH_2), 2.31 (*t*, 4H, CH_2), 2.44 (*sex*, 1H, C*H*), 4.14 (*m*, 2H), 4.30 (*m*, 2H), 5.30 (*m*, 1H). Calc. for $C_{34}H_{64}O_6$: C 71.78; H 11.34; MW 568.85. Found: C 71.9; H 11.4; $(M + NH_4)^+$ 586.

1-(16-Ethyloctadecanoyl)-2,3-dihexanoyl glycerol (**17**) was prepared according to procedure B from 1-(16-ethyloctadecanoyl)-2,3-isopropylidene glycerol (**11**) (8.3 g, 19.5 mmol), pyridine (7.5 mL, 93.7 mmol), and hexanoyl chloride (12.6 g, 93.7 mmol). After flash chromatography (ethyl acetate/petroleum ether, 7.5:92.5) and HPLC (acetonitrile/ethyl acetate, 70:30), **17** (4.4 g, 39%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.82 (*t*, 6H, CH_3), 0.89 (*t*, 6H, CH_3), 1.27 (*m*, 36H, CH_2), 1.62 (*m*, 7H, CH_2, C*H*), 2.30 (*m*, 6H, CH_2), 4.14 (*qu*, 2H), 4.30 (*qu*, 2H), 5.26 (*m*, 1H). Calc. for $C_{35}H_{66}O_6$: C 72.12; H 11.41; MW 582.88. Found: C 73.2; H 11.4; $(M + NH_4)^+$ 600.

1-Hexanoyl-2,3-di-6-propyldecanoyl glycerol (**18**) was prepared according to procedure B from 1-hexanoyl-2,3-isopropylidene glycerol (**1**) (3.33 g, 15.0 mmol), pyridine (2.96 g, 37.5 mmol), and 6-propyldecanoyl chloride (9.77 g, 37.5 mmol). After flash chromatography (heptane/ethyl acetate, 88:12) and HPLC (acetonitrile/ethyl acetate/dichloromethane, 80:10:10), **18** (3.83 g, 40%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*m*, 15H, CH_3), 1.25 (*m*, 40H, CH_2), 1.60 (*m*, 8H, CH_2, C*H*), 2.31 (*m*, 6H, CH_2), 4.15 (*qu*, 2H), 4.30 (*qu*, 2H), 5.30 (*m*, 1H). Calc. for $C_{39}H_{74}O_6$: C 73.30; H 11.67; MW 638.98. Found: C 74.0; H 11.7; $(M + NH_4)^+$ 656.

1-(6-Propyldodecanoyl)-2,3-didecanoyl glycerol (**19**) was prepared according to procedure B from 1-(6-propyldodecanoyl)-2,3-isopropylidene glycerol (**7**) (5.34 g, 15.0 mmol), pyridine (2.96 g, 37.5 mmol), and 6-propyldodecanoyl chloride (8.19 g, 37.5 mmol). After flash chromatography (heptane/ethyl acetate, 88:12), **19** (3.71 g, 38%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*m*, 12H, CH_3), 1.25 (*s*, 50H, CH_2), 1.60 (*m*, 7H, CH_2, C*H*), 2.31 (*sex*, 6H, CH_2), 4.14 (*qu*, 2H), 4.30 (*qu*, 2H), 5.27 (*m*, 1H). Calc. for $C_{42}H_{80}O_6$: C 74.06; H 11.84; MW 681.06. Found: C 74.5; H 11.9; $(M + NH_4)^+$ 698.

1-Dodecanoyl-2,3-di-6-methyldodecanoyl glycerol (**20**) was prepared according to procedure B from 1-dodecanoyl-2,3-isopropylidene glycerol (**6**) (3.67 g, 11.7 mmol), pyridine (3.04 g, 38.5 mmol), and 6-methyldodecanoyl chloride (8.90 g, 38.3 mmol). After flash chromatography (heptane/ethyl acetate, 88:12) and HPLC (acetonitrile/ethyl acetate/dichloromethane, 85:10:5), **20** (5.79 g, 74%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.83 (*d*, 6H, CH_3), 0.88 (*t*, 9H, CH_3), 1.30 (*s*, 44H, CH_2), 1.60 (*m*, 8H, CH_2, C*H*), 2.32 (*t,t*, 6H, CH_2), 4.14 (*qu*, 2H), 4.30 (*qu*, 2H), 5.27 (*m*, 1H). Calc. for $C_{41}H_{78}O_6$: C 73.82; H 11.79; MW 667.03. Found: C 74.0; H 11.7; $(M + NH_4)^+$ 684.

1-Dodecanoyl-1,2-di-10-methyldodecanoyl glycerol (**21**) was prepared according to procedure B from 1-dodecanoyl-2,3-isopropylidene glycerol (**6**) (4.08 g, 13.0 mmol), pyridine (2.54 mL, 31.5 mmol), and 10-methyldodecanoyl chloride (7.30 g, 31.5 mmol). After flash chromatography (ethyl acetate/pentane, 7:93) and HPLC (methanol/ethyl acetate/2-propanol, 85:10:5), **21** (3.10 g, 36%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.84 (*d*, 6H, CH_3), 0.88 (*t*, 9H, CH_3), 1.28 (*s*, 44H, CH_2), 1.61 (*m*, 8H, CH_2, C*H*), 2.31 (*d,t*, 6H, CH_2), 4.14 (*qu*, 2H), 4.30 (*qu*, 2H), 5.27 (*m*, 1H). Calc. for $C_{42}H_{78}O_6$: C 73.82; H 11.79; MW 667.04. Found: C 74.1; H 11.7; $(M + NH_4)^+$ 684.

1-Dodecanoyl-2,3-di-6-propyldodecanoyl glycerol (**22**) was prepared according to procedure B from 1-dodecanoyl-2,3-isopropylidene glycerol (**6**) (3.55 g, 11.3 mmol), pyridine (2.55 mL, 31.7 mmol), and 6-propyldodecanoyl chloride (7.75 g, 29.8 mmol). After flash chromatography (ethyl acetate/pentane, 8:92) and HPLC (methanol/ethyl acetate, 70:30), **22** (5.28 g, 70%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*m*, 15H, CH_3), 1.25 (*m*, 44H, CH_2), 1.59 (*m*, 8H, CH_2, C*H*), 2.32 (*t,t*, 6H, CH_2), 4.14 (*qu*, 2H), 4.30 (*qu*, 2H), 5.27 (*m*, 1H). Calc. for $C_{45}H_{86}O_6$: C 74.74; H 11.99; MW 723.14. Found: C 75.3; 12.1; $(M + NH_4)^+$ 740.

1-Dodecanoyl-2,3-di-9-pentyloctadecanoyl glycerol (**23**) was prepared according to procedure B (the reaction time was prolonged to 6 d) from 1-dodecanoyl-2,3-isopropylidene glycerol (**6**) (2.49 g, 8.3 mmol), pyridine (1.79 mL, 22.3 mmol), and 9-pentyloctadecanoyl chloride (8.30 g, 22.3 mmol). After flash chromatography (ethyl acetate/pentane, 8:92) and HPLC (methanol/ethyl acetate/2-propanol, 45:45:10) **23** (4.80 g, 80%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 15H, CH_3), 1.30 (*m*, 84H, CH_2), 1.61 (*m*, 8H, CH_2, C*H*), 2.31 (*d,t*, 6H, CH_2), 4.14 (*qu*, 2H), 4.30 (*qu*, 2H), 5.26 (*m*, 1H). Calc. for $C_{61}H_{118}O_6$: C 77.343; H 12.55; MW 947.55. Found: C 77.5; H 12.6; $(M + NH_4)^+$ 964.

1-(2-Methylhexanoyl-2,3-dioctadecanoyl glycerol (**24**) was prepared according to procedure B from 1-(2-methylhexanoyl)-2,3-isopropylidene glycerol (**2**) (4.80 g, 19.7 mmol), pyridine (5.60 mL, 69.1 mmol), and octadecanoyl chloride (20.9 g, 69.1 mmol). After flash chromatography (diethyl ether/pentane, 10:90) and HPLC (methanol/chloroform, 70:30), **24** (6.50 g, 45%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*m*, 9H, CH_3), 1.14 (*d, d*, 3H, CH_3), 1.28 (*s*, 60H, CH_2), 1.61 (*m*, 6H, CH_2), 2.31 (*t*, 4H, CH_2), 2.45 (*sex*, 1H, C*H*), 4.14 (*oct*, 2H), 4.31 (*m*, 2H), 5.30 (*m*, 1H). Calc. for $C_{46}H_{88}O_6$: C 74.94; H 12.03; MW 737.16. Found: C 75.5; H 12.5; $(M + NH_4)^+$ 754.

1-(16-Methyloctadecanoyl)-2,3-didodecanoyl glycerol (**25**) was prepared according to procedure B from 1-(16-methyloctadecanoyl)-2,3-isopropylidene glycerol (**10**) (1.50 g, 3.64 mmol), pyridine (1.01 g, 12.74 mmol), and dodecanoyl chloride (2.78 g, 12.74 mmol). After flash chromatography (heptane/ethyl acetate, 88:12), **25** (1.88 g, 70%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.84 (*d*, 3H, CH_3), 0.88 (*t*, 9H, CH_3), 1.30 (*s*, 58H, CH_2), 1.61 (*m*, 7H, CH_2, C*H*), 2.31 (*m*, 6H, CH_2), 4.14 (*qu*, 2H), 4.30 (*qu*, 2H), 5.30 (M, 1H). Calc. for $C_{46}H_{88}O_6$: C 74.94; H 12.03; MW 737.16. Found: C 75.5; H 12.2; $(M + NH_4)^+$ 754.

1-(4-Ethylhexanoyl)-2,3-dioctadecanoyl glycerol (**26**) was prepared according to procedure B from 1-(4-ethylhexanoyl)-2,3-isopropylidene glycerol (**4**) (3.40 g, 13.3 mmol), pyridine (2.70 mL, 34.1 mmol), and octadecanoyl chloride (10.2 g, 33.8 mmol). After flash chromatography (ethyl acetate/heptane, 7:93) and HPLC (methanol/ethyl acetate/2-propanol, 70:20:10), **26** (3.21 g, 32%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.86 (*m*, 12H, CH_3), 1.25 (*s*, 60H, CH_2), 1.59 (*m*, 7H, CH_2, C*H*), 2.30 (*m*, 6H, CH_2), 4.14 (*qu*, 2H), 4.30 (*qu*, 2H), 5.30 (*m*, 1H). Calc. for $C_{47}H_{90}O_6$: C 75.14; H 12.08; MW 751.19. Found: C 74.9; H 12.5; $(M + NH_4)^+$ 768.

1-Dodecanoyl-2,3-di-2-pentyldodecanoyl glycerol (**27**) was prepared according to procedure B from 1-dodecanoyl-2,3-isopropylidene glycerol (**6**) (4.08 g, 13.0 mmol), pyridine (2.54 mL, 31.5 mmol), and 2-pentyldodecanoyl chloride (9.09 g, 31.5 mmol). After flash chromatography (ethyl acetate/pentane, 7:92) and HPLC (methanol/ethyl acetate, 65:35), **27** (4.05 g, 40%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 15H, CH_3), 1.25 (*s*, 60H, CH_2), 1.59 (*m*, 10H, CH_2), 2.29 (*t*, 2H, CH_2), 2.32 (*m*, 2H, C*H*), 4.12 (*oct*, 2H), 4.32 (*oct*, 2H), 5.28 (*m*, 1H). Calc. for $C_{49}H_{94}O_6$: C 75.52; H 12.16; MW 779.43: Found: C 76.1; H 11.9; $(M + NH_4)^+$ 796.

1-Octadecanoyl-2,3-di-6-propyldodecanoyl glycerol (**28**) was prepared according to procedure B from 1-octadecanoyl-2,3-isopropylidene glycerol (**8**) (5.17 g, 13 mmol), pyridine (2.54 mL, 31.5 mmol), and 6-propyldodecanoyl chloride (8.02 g, 31.5 mmol). After flash chromatography (ethyl acetate/pentane, 7:93) and HPLC (methanol/ethyl acetate/2-propanol, 70:20:10), **28** (4.73 g, 45%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.87 (*m*, 15H, CH_3), 1.25 (*s*, 64H, CH_2), 1.60 (*m*, 8H, CH_2, C*H*), 2.32 (*t,t,t*, 6H, CH_2), 4.14 (*qu*, 2H), 4.30 (*qu*, 2H), 5.30 (*m*, 1H). Calc. for $C_{51}H_{98}O_6$: C 75.87; H 12.24; MW 807.29. Found: C 76.3; H 11.3; $(M + NH_4)^+$ 824.

1-Dodecanoyl-2,3-di-6-hexyldodecanoyl glycerol (**29**) was prepared according to procedure B from 1-dodecanoyl-2,3-isopropylidene glycerol (**6**) (4.24 g, 13.5 mmol), pyridine (3.20 g, 40.5 mmol), and 6-hexyldodecanoyl chloride (12.28 g, 40.50 mmol). After flash chromatography (heptane/ethyl acetate, 88:12) and HPLC (acetonitrile/ethyl acetate/dichloromethane, 73:23:4), **29** (5.44 g, 50%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 15H, CH_3), 1.25 (*s*, 64H, CH_2), 1.59 (*m*, 8H, CH_2, C*H*), 2.32 (*t,t,t*, 6H, CH_2), 4.14 (*qu*, 2H), 4.30 (*qu*,

2H), 5.25 (*m*, 1H). Calc. for $C_{51}H_{98}O_6$: C 75.87; H 12.24; MW 807.29. Found: 76.7; H 12.4; $(M + NH_4)^+$ 824.

1-(6-Propyldodecanoyl)-2,3-dioctadecanoyl glycerol (**30**) was prepared according to procedure B from 1-(6-propyldodecanoyl-2,3-isopropylidene glycerol (**7**) (4.00 g, 11.2 mmol), pyridine (3.13 g, 39.2 mmol), and octanoyl chloride (11.86 g, 39.20 mmol). After flash chromatography (heptane/ethyl acetate, 88:12), **30** (4.75 g, 50.0%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.82 (*m*, 12H, C*H*$_3$), 1.25 (*s*, 74H, C*H*$_2$), 1.61 (*m*, 7H, C*H*$_2$, C*H*), 2.30 (*t,t*, 6H, C*H*$_2$), 4.14 (*qu*, 2H), 4.30 (*qu*, 2H), 5.30 (*m*, 1H). Calc. for $C_{54}H_{104}O_6$: C 76.37; H 12.34; MW 849.37. Found: C 76.6; H 12.6; $(M + NH_4)^+$ 866.

1-(16-Methyloctadecanoyl)-2,3-dioctadecanoyl glycerol (**31**) was prepared according to procedure B from 1-(16-methyloctadecanoyl)-2,3-isopropylidene glycerol (**10**) (1.70 g, 4.00 mmol), pyridine (1.16 mL, 14.8 mmol), and octadecanoyl chloride (4.47 g, 14.8 mmol). After flash chromatography (ether/pentane, 1:1) and HPLC (chloroform/acetonitrile/2-propanol, 54:44:2), **31** was obtained with m.p. 56.5–58.0°C. ^{1}H NMR ($CDCl_3$) δ 0.83 (*m*, 12H, C*H*$_3$), 1.25 (83H, C*H*$_2$, C*H*) 1.57 (*m*, 6H, C*H*$_2$), 2.31 (*m*, 6H, C*H*$_2$), 4.15 (*qv*, 2H, OC*H*H), 4.22 (*qv*, 2H, OCH*H*), 5.27 (*m*, 1H, OC*H*). Peak matching on $(M + NH_4)^+$. Calc. for $C_{58}H_{116}NO_6$: 922.8803. Found: 922.8834.

1-(2-Hexadecyloctadecanoyl)-2,3-dioctadecanoyl glycerol (**32**) was prepared according to procedure B from 1-(2-hexadecyloctadecanoyl)-2,3-isopropylidene glycerol (**12**) (4.40 g, 7.10 mmol), pyridine (3.20 mL, 39.5 mmol), and octadecanoyl chloride (11.9 g, 39.5 mmol). After flash chromatography (pentane/diethyl ether, 90:10) and HPLC (acetonitrile/dichloromethane, 70:30), **32** (4.70 g, 59.0%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 12H, C*H*$_3$), 1.25 (*s*, 112H, C*H*$_2$), 1.60 (*m*, 8H, C*H*$_2$), 2.30 (*t,t*, 4H, C*H*$_2$), 2.32 (*m*, 1H, C*H*), 4.14 (*oct*, 2H), 4.31 (*hept*, 2H), 5.30 (*m*, 1H). Calc. for $C_{73}H_{142}O_6$: C 78.57; H 12.38; MW 1115.88. Found: C 79.1; H 13.1; $(M + NH_4)^+$ 1132.

Tris(2-methylhexanoyl) glycerol (**33**) was prepared according to procedure C from 2-hexanoic acid (21.0 g, 169 mmol), glycerol (4.30 g, 47.0 mmol), and 4-toluenesulfonic acid in toluene (40 mL). After workup and chromatography (petroleum ether/diethyl ether, 95:5), **33** (6.7 g, 34%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 9H, C*H*$_3$), 1.14 (*d*, 9H, C*H*), 1.28 (*m*, 12H, C*H*$_2$), 1.64 (*m*, 6H, C*H*$_2$), 2.44 (*m*, 3H, C*H*), 4.14 (*m*, 2H, C*H*H), 4.32 (*m*, 2H, CH*H*), 5.30 (*m*, 1H, C*H*). MS peak matching on $(M + NH_4)^+$. Calc. for $C_{24}H_{46}NO_6$: 446. Found 446.

Tris(4-methylhexanoyl) glycerol (**34**) was prepared according to procedure C from 4-methylhexanoic acid (15.6 g, 120 mmol), glycerol (3.1 g, 34 mmol), and 4-toluenesulfonic acid (0.5 g, 3.0 mmol) in toluene (40 mL). Workup after a reaction time of 130 h and chromatography (petroleum ether/diethyl ether, 95:5) gave **34** (6.3 g, 44%). ^{1}H NMR ($CDCl_3$) δ 0.88 (*m*, 18H, C*H*$_3$), 1.35 (*m*, 9H, C*H*$_2$, C*H*), 1.65 (*m*, 6H, CH$_2$), 2.30 (*m*, 6H, C*H*$_2$), 4.16 (*qu*, 2H), 4.30 (*qu*, 2H), 5.30 (*m*, 1H). Calc. for $C_{24}H_{44}O_6$: C 67.25; H 10.35; MW 428.59. Found: C 68.1; H 10.4; $(M + NH_4)^+$ 446.

Tris(4-ethylhexanoyl) glycerol (**35**) was prepared according to procedure D from glycerol (2.10 g, 22.4 mmol), pyridine (8.10 mL, 101 mmol), and 4-ethylhexanoyl chloride (16.4 g, 101 mmol) in dichloromethane (150 mL). After workup and flash chromatography (ethyl acetate/petroleum ether), **35** (1.5 g, 14%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.85 (*t*, 18H, C*H*$_3$), 1.27 (*m*, 9H, C*H*$_2$, C*H*), 1.60 (*m*, 6H, C*H*$_2$), 2.30 (*m*, 6H, C*H*$_2$), 4.15 (*qu*, 2H), 4.30 (*qu*, 2H), 5.30 (m, 1H). Calc. for $C_{27}H_{50}O_6$: C 68.90; H 10.71; MW 470.67. Found: C 68.7; H 10.6; $(M + NH_4)^+$ 488.

Tris(2-butylhexanoyl) glycerol (**36**) (1) was prepared according to procedure C from 2-butylhexanoic acid (15.0 g, 87.0 mmol), glycerol (2.2 g, 24 mmol), and 4-toluenesulfonic acid (0.5 g, 3.0 mmol) in toluene (40 mL). Workup after a reaction time of 130 h and chromatography (petroleum ether/diethyl ether, 95:5) gave **36** (3.8 g, 30%). ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 18H, C*H*$_3$), 1.25 (*m*, 24H, C*H*$_2$), 1.45 (*m*, 6H, C*H*$_2$), 1.60 (*m*, 6H, C*H*$_2$), 2.33 (*m*, 3H, C*H*), 4.10 (*qu*, 2H), 4.35 (*qu*, 2H), 5.28 (*m*, 1H).

Tris(6-propyldodecanoyl) glycerol (**37**) was prepared according to procedure D from glycerol (1.20 g, 13.0 mmol), pyridine (4.80 m, 58.5 mmol), and 6-propyldodecanoyl chloride (15.2 g, 58.5 mmol) in dichloromethane (80 mL). After workup and flash chromatography (ethyl acetate/petroleum ether), **37** (6.5 g, 65%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t,t*, 18H, C*H*$_3$), 1.23 (*m*, 54H, C*H*$_2$), 1.59 (*m*, 9H, C*H*$_2$), 2.32 (*t,t*, C*H*$_2$), 4.14 (*qu*, 2H), 4.30 (*qu*, 2H), 5.30 (*m*, 1H). Calc. for $C_{48}H_{92}O_6$: C 75.34; H 12.27; MW 765.22. Found: C 76.0; H 12.1; $(M + NH_4)^+$ 782.

Tris(2-methyloctadecanoyl) glycerol (**38**) was prepared according to procedure D from glycerol (0.8 g, 86 mmol), pyridine (3.10 mL, 38.5 mmol), and 2-methyloctadecanoyl chloride (12.2 g, 38.5 mmol) in dichloromethane (80 mL). After workup and flash chromatography (ethyl acetate/petroleum ether), **38** (3.8 g, 47%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 9H, C*H*$_3$), 1.14 (*d,d*, 9H, C*H*$_3$), 1.26 (*s*, 84H, C*H*$_2$), 1.63 (*m*, 6H, C*H*$_2$), 2.43 (*sex*, 3H, C*H*), 4.13 (*m*, 2H), 4.32 (*m*, 2H), 5.30 (*m*, 1H). Calc. for $C_{60}H_{116}O_6$: C 77.19; H 12.53; MW 933.53. Found: C 77.4; H 12.8; $(M + NH_4)^+$ 950.

Tris(16-methyloctadecanoyl) glycerol (**39**) was prepared according to procedure D from glycerol (1.0 g, 10.7 mmol), pyridine (3.90 g, 48.1 mmol), and 16-methyloctadecanoyl chloride (15.2 g, 48.1 mmol) in dichloromethane (80 mL). After workup and flash chromatography (ethyl acetate/petroleum ether), **39** (4.9 g, 49%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.84 (*d*, 9H, C*H*$_3$), 0.85 (*t*, 9H, C*H*$_3$), 1.25 (*s*, 78H, C*H*$_2$), 1.61 (*m*, 9H, C*H*$_2$, C*H*), 2.31 (*t,t*, 6H, C*H*$_2$), 4.14 (*qu*, 2H), 4.30 (*qu*, 2H), 5.27 (*m*, 1H). Calc. for $C_{60}H_{116}O_6$: C 77.19; H 12.53; MW 933.53. Found: C 77.1; H 12.5; $(M + NH_4)^+$ 950.

Tris(16-ethyloctadecanoyl) glycerol (**40**) was prepared according to procedure D from glycerol (1.10 g, 1.09 mmol), pyridine (0.34 mL, 4.24 mmol), and 16-ethyloctadecanoyl chloride (1.40 g, 4.24 mmol) in dichloromethane (7.0 mL). After work up and flash chromatography, **40** (0.54 g, 51.0%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.83 (*t*, 18H, C*H*$_3$), 1.26 (*s*, 84H, C*H*$_2$), 1.27 (*t*, 3H, C*H*), 1.61 (*m*, 6H, C*H*$_2$), 2.31

(*t*, 5H, CH_2), 2.32 (*t*, 2H, CH_2), 4.15 (*qu*, 2H), 4.29 (*qu*, 2H), 5.27 (*m*, 1H). Calc. for $C_{63}H_{122}O_6$: C 77.55; H 12.61; MW 975.61. Found: C 78.3; H 12.8; $(M + NH_4)^+$ 992.

RESULTS AND DISCUSSION

The racemic 1-acyl-2,3-isopropylidene glycerols (**1–13**) were prepared from the appropriate acyl chloride and 1,2-isopropylidene glycerol in pyridine and toluene (7). The presence of pyridine prevents migration of the sensitive isopropylidene ketal (8). Deprotection of the isopropylidene group into the monoacylglycerols can be accomplished by a number of acidic procedures, but since monoacylglycerols are readily isomerized by acid, alkali and heat, the reaction conditions are very important. Deprotection without any acyl migration has been achieved with dimethyl boron bromide at –50°C (9). We chose the less expensive method using boric acid in 2-methoxyethanol at 100°C, which also worked satisfactorily (10). The monoacylglycerols were acylated immediately in the manner described above in order to avoid isomerization. The triacylglycerols so obtained (**14–32**) are given in Scheme 1. The monoacid triacylglycerols (**33–40**) in Scheme 2 were prepared either from glycerol and acyl chloride in pyridine and toluene or in an acid-catalyzed reaction between glycerol and the branched acid in toluene (1).

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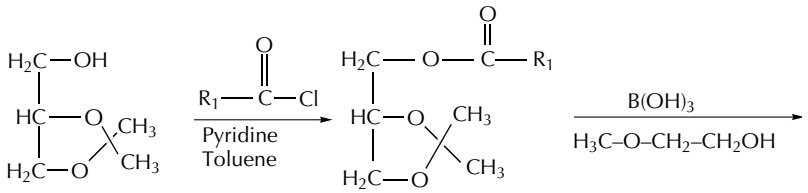
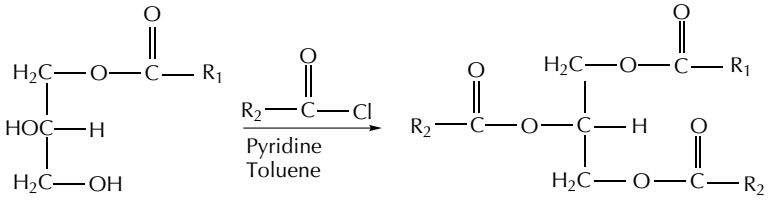


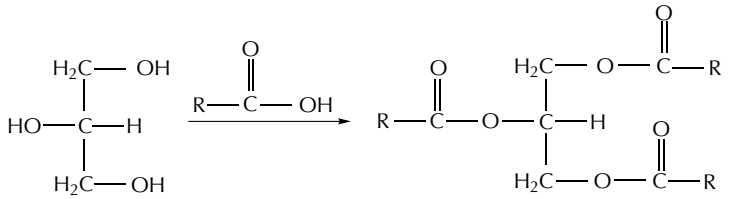
1–13



14–32

	R_1	R_2
14	2-butylpentyl	pentyl
15	undecyl	3-ethylpentyl
16	2-methylheptyl	pentyl
17	16-ethylheptadecyl	pentyl
18	pentyl	6-propylnonyl
19	6-propylundecyl	undecyl
20	undecyl	6-methylundecyl
21	undecyl	10-methylundecyl
22	undecyl	6-propylundecyl
23	undecyl	9-pentylundecyl
24	2-methylpentyl	heptadecyl
25	16-methylheptadecyl	undecyl
26	4-ethylpentyl	heptadecyl
27	undecyl	2-pentylundecyl
28	heptadecyl	6-propylundecyl
29	undecyl	6-hexylundecyl
30	6-propyldodecyl	heptadecyl
31	16-methylheptadecyl	heptadecyl
32	2-hexadecylheptadecyl	heptadecyl

SCHEME 1



33–40

R

33 2-methylpentyl
34 4-methylpentyl
35 4-ethylpentyl*
36 2-butylpentyl
37 6-propylundecyl*
38 2-methylheptadecyl*
39 16-methylheptadecyl*
40 16-ethylheptadecyl*

*Prepared from the acyl chlorides

SCHEME 2

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COMMUNICATION

A New Lipid from an Australian Marine Sponge, *Callyspongia* sp.

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ABSTRACT: A specimen of the sponge *Callyspongia* sp. collected off the coast of New South Wales, Australia, has yielded the novel lipid (6*Z*,9*Z*,12*Z*,15*Z*)-1,6,9,12,15-octadecapenten-3-one, together with (4*Z*,7*Z*,10*Z*,13*Z*)-4,7,10,13-hexadecatetraenoic acid.

Lipids 32, 675–677 (1997).

Marine organisms are an established source of novel secondary metabolites and are increasingly recognized as a source of unusual lipids. In this context, a number of novel polyunsaturated fatty acids have been isolated and reported to possess interesting biological properties [enzyme inhibitory (1), ichthyotoxic (2), allelopathic (3–5), antibiotic (6), etc.]. For example, the acetylenic fatty acid (**1**) from Pacific Ocean and Caribbean Sea collections of the red alga *Liagora farinosa* (7) has been described as a potent inhibitor of bee venom-derived phospholipase A_2 (100% inhibition; 5.7 mM) (1), while the allelopathic fatty acid (6*Z*,9*Z*,12*Z*,15*Z*)-6,9,12,15-octadecatetraenoic acid (**2**) was isolated from the commercially available edible marine brown alga *Cladosiphon okamuranus* (5). The polyunsaturated fatty acid (4*Z*,7*Z*,10*Z*,13*Z*)-4,7,10,13-hexadecatetraenoic acid (**3**) first reported from the alga *Scenedesmus obiliquas* (8) was reisolated from a Japanese freshwater chlorophyte *Pediastrum* sp. (9) at which time it was shown to inhibit the development of starfish embryos. A closely related polyunsaturated fatty acid methyl ester (**4**) has since been reported from a Japanese soft coral *Xenia* sp. (10). Sponges have also been a source of structurally unusual acetogenins, such as debromorenierin-1 (**5**) from an Italian marine sponge, *Reniera fulva* (11).

In this report we describe the isolation, characterization, and identification of a new lipid, (6*Z*,9*Z*,12*Z*,15*Z*)-1,6,9,12,15-octadecapenten-3-one (**6**), as well as the previously reported polyunsaturated fatty acid, (4*Z*,7*Z*,10*Z*,13*Z*)-4,7,10,13-hexadecatetraenoic acid (**3**), from an Australian sponge, *Callyspongia* sp.

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Abbreviations: CI, chemical ionization; EI, electron impact; GC, gas chromatography; HPLC, high-pressure liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; UV/vis, ultraviolet/visible.

EXPERIMENTAL PROCEDURES

General. All solvents were redistilled before use. Rapid silica filtrations were performed by using Kieselgel 60 silica. Ultraviolet (UV) absorption spectra (λ_{max}) were obtained on a Varian Superscan 3 UV/visible (UV/vis) spectrophotometer (Palo Alto, CA), while infrared (IR) spectra (ν_{max}) were obtained on either a Perkin-Elmer 983G or Perkin-Elmer 1600 Fourier transform infrared spectrometer (Norwalk, CT). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra, as well as two-dimensional NMR experiments, were performed on a Varian Unity 300 spectrometer in the solvents indicated, with spectra referenced to residual ^1H signals in deuterated solvents. Low- and high-resolution electron impact (EI) mass spectra were recorded on either a V.G. Micromass 7070F (Cheshire, England) or a Jeol AX-505H mass spectrometer (Tokyo, Japan), with chemical ionization (CI) mass spectra being recorded on the latter instrument. High-pressure liquid chromatography (HPLC) was performed by means of an ISCO 2350 solvent delivery system (Lincoln, NE) equipped with a Rheodyne injector, Waters 401 differential refractometer (Milford, MA), and Spectra Physics 200 programmable UV/vis wavelength detector (San Jose, CA), and recorded on a Data Acquisition Plotting and Analysis (DAPA) (Perth, Australia) package operating on an Ipex 286PC unit under DOS 5.0 (Melbourne, Australia).

Analytical capillary GC was performed on a Hewlett-Packard 5890 gas chromatograph (Wilmington, DE) equipped and recorded on a data acquisition package (Turbochrom 4; San Jose, CA) operating on a Digital DEC PC under DOS 6.2. The column used was a BP-1 (15 m by 0.22 mm and film thickness 0.25 mm). GC EI mass spectrometry (MS) was carried out on a Jeol AX-505H mass spectrometer equipped with the above column using the following temperature program: 40°C (2 min), followed by ramping at 10°C/min to 270°C (5 min), carrier gas flow 2–3 mL/min, injector temperature 250°C, detector temperature 350°C.

Collection, extraction, and isolation. A specimen of a *Callyspongia* sp. (class Demospongiae: order Haplosclerida: family Callyspongiidae) (12 g extracted dry weight) (Museum of Victoria registry number F77031) was collected by SCUBA (−12 m) off Durras, New South Wales, Australia

(35°40.1′S 150°18.5′E). This sponge exhibits a massive-bulbous growth form. Color in life is pale lilac-mauve and beige when preserved in ethanol. The texture is compressible-spongy yet fibrous and reasonably tough to tear. Oscules are scattered, conspicuous, and often raised with a distinct lip. The sponge surface is transparent with fiber reticulation visible at the surface, optically smooth, but minutely conulose. Structural megascleres are vestigial fusiform oxeas with telescoped ends of dimensions 85–115 µm × 2.5 µm. The peripheral skeleton or ectosome is a tangential isodictyal reticulation of primary multispicular, secondary bispicular and tertiary unispicular fibers with choaonosomal primary fibers occasionally protruding in support of surface conules. The choanosomal skeleton exhibits a wide-spaced isodictyal reticulation of primary fibers 50–75 µm in width, cored by 10–20 oxeas and connecting secondary fibers cored by 2–5 oxeas, with unispicular tertiary fibers becoming more common in the subectosomal region. Spongin is light. Collagen in the mesohyl is abundant and scattered lightly with single interstitial spicules arranged isodictyally.

The fresh sponge was diced, steeped in ethanol, and stored in the dark at –20°C. The crude ethanol extract of this sponge was concentrated under reduced pressure and the CH_2Cl_2-soluble portion subjected to rapid silica filtration [20% stepwise elution from petroleum spirits (60–80°C) to EtOAc] followed by normal-phase HPLC (2.0 mL/min, 20% EtOAc/petroleum spirits, Phenomenex Spherex 5 µ Si 250 × 10 mm column; Torrance, CA) to yield (6*Z*,9*Z*,12*Z*,15*Z*)-1,6,9,12,15-octadecapenten-3-one (**6**) (7 mg, 0.05%) and (4*Z*,7*Z*,10*Z*,13*Z*)-4,7,10,13-hexadecatetraenoic acid (**3**) (4 mg, 0.03%).

*(6Z,9Z,12Z,15Z)-1,6,9,12,15-octadecapenten-3-one (***6***).* A stable colorless oil. ν_{max} (film) 1697, 1678 cm^{-1}; λ_{max} (EtOH) 203 (ε = 22,000) nm; ^1H (300 MHz, $CDCl_3$) δ 6.36, *dd, J* = 11.0, 17.6 Hz, H 2; 6.22, *dd, J* = 1.3, 17.6 Hz, H 1_{trans}; 5.83, *dd, J* = 1.3, 11.0 Hz, H 1_{cis}; 5.38, *bm*, H 6, H 7, H 9, H 10, H 12, H 13, H 15, H 16; 2.83, *bm*, $(H\ 8)_2$, $(H\ 11)_2$, $(H\ 14)_2$; 2.65, *t, J* = 7.3 Hz, $(H\ 4)_2$; 2.39, *dt, J* = 6.9, 7.3 Hz, $(H\ 5)_2$; 2.07 *dq, J* = 6.9, 7.3 Hz, $(H\ 17)_2$; 0.97, *t, J* = 7.3 Hz, $(H\ 18)_3$; ^{13}C (75 MHz, $CDCl_3$) δ 200.1, *s*, C 3; 136.5, *d*, C 2; 132.0, *d*, C 16[1]; 129.0, *d*, C 6[1]; 128.6, *d*[1], C 15; 128.3, 2*d*, C 9 and C 10[1]; 128.1, *t*, C 1; 128.0, *d*, C 7[1]; 127.8, *d*, C 12[1]; 126.9, *d*, C 13[1]; 39.4, *t*, C 4; 25.6, 2*d*, C 8 and C 11[2]; 25.5, *t*, C 14[2]; 21.7, *t*, C 5[3]; 20.6, *t*, C 17[3]; 14.3, *q*, C 18; EI–MS (70 eV) *m/z* (%) 258 (M^+, 1), 256 (1), 229 (1), 203 (1), 189 (2), 188 (4), 175 (2), 149 (4), 135 (8), 131 (11), 119 (24), 105 (26), 95 (27), 91 (63), 79 (100), 69 (11), 55 (99); CI–MS (Methane) *m/z* (%) 258 (M^+, 7), 203 (8), 189 (10), 188 (15), 175 (8), 149 (16), 135 (24), 133 (26), 119 (44), 108 (56), 95 (44), 91 (87), 69 (35), 79 (100); high-resolution EI–MS *m/z* 258.1972 ($C_{18}H_{26}O$ requires 258.1983); *m/z* 203.1794 ($C_{15}H_{23}^+$ requires 203.1799); *m/z* 189.1637 ($C_{14}H_{21}^+$ requires 189.1643). (Note: [1,2,3] Assignments may be interchanged.)

RESULTS AND DISCUSSION

The crude ethanol extract of a specimen of *Callyspongia* sp. collected by SCUBA off the coast of New South Wales, Australia, was concentrated and subjected to the purification procedure as outlined in the Experimental Procedures section to yield two lipid-soluble compounds, (6*Z*,9*Z*,12*Z*,15*Z*)1,6,9,12,15-octadecapenten-3-one (**6**) and (4*Z*,7*Z*, 10*Z*,13*Z*)4,7,10,13-hexadecatetraenoic acid (**3**).

Compound (**6**) was isolated by HPLC as a stable, colorless oil, and its purity confirmed by GC analysis. Although the EI mass spectrum of (**6**) revealed only a very weak molecular ion (<1%), CI mass spectral analysis established the molecular formula ($C_{18}H_{26}O$, Δmmu − 1.1) to require six degrees of unsaturation.

The presence of a conjugated ketone functionality was supported by an IR absorption (1697 cm^{-1}) and a characteristic ^{13}C NMR resonance (200.1, s). Further examination of the ^1H and ^{13}C NMR data for (**6**) revealed the presence of an isolated terminal double bond [^1H: δ 6.36 (*dd, J* = 11.0, 17.6 Hz), 6.22 (*dd, J* = 1.3, 17.6 Hz) and 5.83 (*dd, J* = 1.3, 11.0 Hz); ^{13}C: 128.1 (*t*) and 136.5 (*d*) ppm], four 1,2-disubstituted double bonds [^1H: δ 5.38 (*bm*); ^{13}C: 132.0 (*d*), 129.0 (*d*), 128.6 (*d*), 128.3 (*d*), 128.3 (*d*), 128.0 (*d*), 127.8 (*d*), 126.9 (*d*) ppm] and three bisallylic methylenes [^1H: δ 2.83 (*bm*, 6H)]. These latter observations together with resonances for a primary methyl group β to a double bond [^1H: δ 0.97 (*bt*)] established the C_6 to C_{18} structure fragment. Furthermore, the deshielded ^{13}C NMR chemical shifts for the bisallylic [^{13}C: 25.5 (*t*) and 25.6 (2*t*) ppm] and allylic [^{13}C: 21.7 (*t*) and 20.6 (*t*) ppm] methylene carbons were supportive of an all *Z* geometry (12–14). Detailed examination of the COSY NMR data provided correlations (see Fig. 1) to support the complete struc-

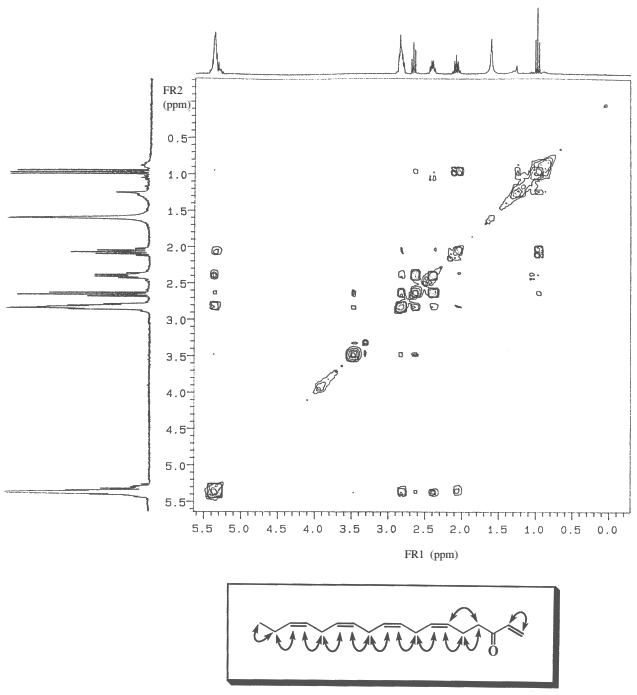
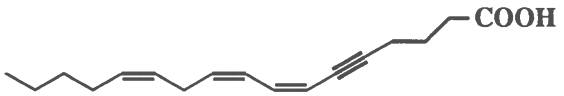


FIG. 1. Selected COSY NMR correlations for (**6**).

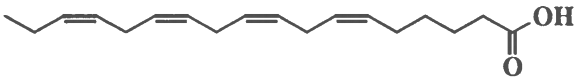
ture as shown, while diagnostic ions were observed in the EI and CI mass spectra (*m/z* 203 and 189).

Compound (**3**) was isolated by HPLC as an unstable, colorless oil, and its purity confirmed by GC analysis. The ^{1}H NMR, IR, and mass spectral data for (**3**) were found to be consistent with that reported for (4*Z*,7*Z*,10*Z*,13*Z*)4,7,10,13-hexadecatetraenoic acid (10,15,16).

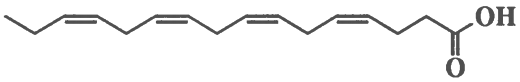
Although the ecological role of the new lipid (6*Z*,9*Z*,12*Z*,15*Z*)-1,6,9,12,15-octadecapenten-3-one (**6**) or the known polyunsaturated fatty acid (4*Z*,7*Z*,10*Z*,13*Z*)4,7,10,13-hexadecatetraenoic acid (**3**) cannot be defined, it is possible to speculate. Filter-feeding organisms such as sponges are susceptible to overgrowth and as with the alga mentioned earlier in this report polyunsaturated lipids such as (**3**) may afford a level of chemical defense and/or the capacity to overgrow adjacent organisms (Scheme 1).



(**1**)

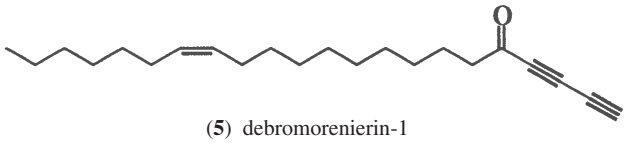


(**2**) (6*Z*,9*Z*,12*Z*,15*Z*)-6,9,12,15-octadecatetraenoic acid

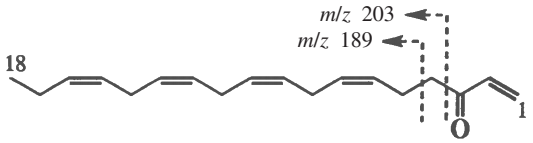


(**3**) (4*Z*,7*Z*,10*Z*,13*Z*)-4,7,10,13-hexadecatetraenoic acid

(**4**)



(**5**) debromorenierin-1



(**6**) (6*Z*,9*Z*,12*Z*,15*Z*)-1,6,9,12,15-octadecapenten-3-one

SCHEME 1

ACKNOWLEDGMENTS

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Gas–Liquid Chromatographic Properties of Positional Isomers of Methyl Thia, Selena, and Tellura Laurate Analogs

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ABSTRACT: Gas–liquid chromatographic analyses of three complete series of synthetic positional isomers of methyl thia, selena, and tellura laurate analogs were carried on a nonpolar (SE-30) and a polar (SP-2330) stationary phase. The average ECL (equivalent chain length) values of the thia, selena, and tellura laurate on SE-30 stationary phase were 13.8, 14.8, and 15.7, respectively, while on SP-2330 the average values for the same series were 17.1, 19.0, and 19.1, respectively. Positional isomers with the heteroatom at the 2-position exhibited the lowest ECL values, while those with the heteroatom at the ω-1 position gave the highest ECL values and were readily separated from the other positional isomers of the same series of analogs by this technique.

Lipids 32, 679–681 (1997).

Corey *et al.* (1) have synthesized 7-thia-arachidonic acid, which has been found to be a potent irreversible inhibitor of leukotriene (also known as lipoxin) biosynthesis. This observation suggests a possible means of control of inflammatory diseases caused by an overproduction of leukotrienes in the human body (2). In the field of fatty acid chemistry, only a small number of long-chain fatty acids containing a sulfur atom have been synthesized in order to study their chemical, physical, or biological properties (3–6). Pascal *et al.* (7,8) have prepared the 9- and 10-thiastearic acid isomers and have reported on the inhibitory effects of these fatty acid analogs on cyclopropanoid fatty acid biosynthesis and on the growth of a protozoan species, *Crithidia fasciculata*. Buist's group (9) has been engaged in the study of the metabolism of thiastearic acids by yeast cells, *Saccharomyces cerevisiae*, and have noted a highly chemo-, regio-, and stereoselective introduction of a (*Z*)-olefinic bond into such sulfur-containing fatty acid analogs. Recently the inhibition of protozoan cyclopropane fatty acid synthetase by phosphatidylethanolamines containing thia fatty acid moieties has been reported (10). Metabolism of 3-thia fatty acids in rat liver and other tissues has also been investigated (11).

Fatty acids containing a selenium or tellurium atom are not found in nature. A study of some synthetic selena-alkanoic acids has been reported by Schwarz and Fredga (12), which shows that such selena analogs prevent dietary liver necrosis in rats. Results of the antimicrobial action of selena-alkanoic acids against *Streptococcus pyogenes* show that the reactivity is more effective in the C_{14} acid type than the corresponding C_{15} or C_{16} selena homologs. The position of the selenium atom in the alkyl chain also appears to affect the antimicrobial potency of such derivatives (13). Radioactive-labeled selena fatty acids have been used as myocardial imaging agents (14–16). Success has been achieved with fatty acids containing radioactive ^{125}I and nonradioactive Te atoms as imaging agent, which provide a rapid and pronounced myocardial uptake agent with the advantage of prolonging the myocardial retention and lowering the *in vivo* deiodination periods (17).

In view of these important developments in the chemistry and biochemistry of long-chain fatty acids containing a heteroatom, we have synthesized complete series of thia (18), selena, and tellura (19) fatty acid esters to study their nuclear magnetic resonance spectroscopic and mass spectral properties (20). In this paper we report the gas–liquid chromatographic properties of the entire series of positional isomers of methyl thia, selena, and tellura laurate analogs:

$CH_3(CH_2)_n$-*X*-$(CH_2)_mCOOCH_3$, where $n + m = 9$ and *X* = S, Se, or Te.

MATERIALS AND METHODS

The syntheses of methyl thia, selena, and tellura laurate isomers have been described elsewhere (18,19). Gas chromatographic analysis was carried out on a Hewlett-Packard (model 5890) (Hewlett Packard Inc., Palo Alto, CA) gas chromatograph fitted with a 25-m glass column (0.3 mm diameter, 0.33 μm film thickness, SE-30 stationary phase) or 30-m glass column (0.25 mm diameter, 0.20 μm film thickness, SP-2330 stationary phase). Helium (2 mL flow) was used as the carrier gas at an isothermal column temperature (170°C for SE-30 or 140°C for SP-2330) with a flame-ionization detector. External methyl fatty esters (methyl laurate, myristate, palmitate, and stearate) were used as reference compounds and the ECL (equivalent chain length) (21) values were calculated accordingly.

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Abbreviation: ECL, equivalent chain length.

RESULTS AND DISCUSSION

The results of the gas–liquid chromatographic properties of the positional isomers of methyl thia, selena, and tellura laurate on nonpolar (SE-30) and polar (SP-2330) stationary phases are presented as their ECL values in Table 1. In general, nonpolar stationary phases, such as SE-30, separate compounds according to their molecular weights. The heavier the molecular mass, the longer the retention time. The replacement of a methylene group in the alkyl chain of methyl laurate by either a sulfur, selenium, or tellurium atom increases the molecular weight of the parent fatty ester (methyl laurate) substantially. It would therefore be reasonable to expect a large increase in the retention time for the thia, selena, and tellura analogs as compared to the parent methyl ester (methyl laurate). This change in the retention times was reflected by the high ECL values obtained on the nonpolar stationary phases for these three series of analogs. However, the actual ECL values of these analogs did not reflect a substantial proportional increase of anticipated retention time. Bearing in mind that the atomic mass of a tellurium atom is *ca.* 1.6-fold heavier than a selenium atom, which in turn is *ca.* 2.5-fold heavier than a sulfur atom, while the latter is *ca.* 2.3 times heavier than the mass of a methylene group in methyl laurate, it would be expected from a substance like methyl tellura laurate to give an estimated ECL value of at least 11.0 + 9.1 = 20.1. The value of 9.1 was obtained by dividing the atomic mass of tellurium (average value 127.60) by the mass of a methylene group (14.0) to give a quotient of 9.1. By adding this value to the theoretically remaining value of the retention time of a methyl laurate less one methylene group (i.e., being replaced by a hetero atom, leaves an ECL value = 11.0). However, from the results of the study of the methyl tellura laurate isomers, the highest ECL value obtained (methyl 11-tellura laurate) was 16.3 on SE-30 stationary phase. Similarly, the ECL values of the selena and tellura laurates series were in relative terms lower than the estimated ECL values based on the large molecular weights of the parent fatty ester by substitution of one methylene group by a sulfur or selenium atom. The average ECL values of methyl thia, selena, and tellura laurate analogs were 13.8, 14.8, and 15.7, respectively.

The chromatographic results on the SE-30 stationary phase also showed that the farther the position of the hetero atom is from the methyl ester function, the longer the retention time of the positional isomer on the column. Thus, the 2-positional isomers exhibited the lowest ECL values in each series, while the ECL value of the ω-1 isomers (*viz.* 11-thia, 11-selena, and 11-tellura isomers) furnished the highest ECL values. The low ECL values of the 2-positional isomers were considered to be attributed to the results of the conjugative electronic effects involving the lone pair of electrons of the hetero atom (sulfur, selenium, or tellurium) and the polarizable nature of the C=O bond of the ester function. With the position of the hetero atom becoming increasingly remotely positioned from the ester function, the retention times of the positional isomers of heteroatomic fatty esters increased gradually and reached a maximum value for the 11-isomers in each series.

The results on the polar stationary phase (SP-2330) were of interest, as the result of the conjugative electronic effects involving the lone pair of electrons of the hetero atom (sulfur, selenium, or tellurium) also furnished the lowest ECL value for the 2-positional isomer in each of the series studied. While the selena isomers (average ECL value = 19.0) exhibited a marked increase in retention time over the thia isomers (average ECL value = 17.1), the difference in ECL value of the tellura isomers (average ECL value = 19.1) was very similar to those exhibited by the selena isomers. There appeared to be hardly a difference in polarity between a tellura and a selena analog, despite their differences in atomic mass, when the analysis was conducted on the polar SP-2330 stationary phase. Separation of methyl selena and tellura isomers would therefore be difficult, if not impossible, on a polar stationary phase.

We conclude from this study that separation and possible identification of some of the positional isomers of methyl thia, selena, and tellura laurate isomers by gas–liquid chromatographic analysis is feasible using a combination of polar and nonpolar stationary phases.

TABLE 1
Results (equivalent chain length values) of the Gas–Liquid Chromatographic Analyses of Positional Isomers of Methyl Thia, Selena, and Tellura Laurate Analogs on SE-30 and SP-2330 Stationary Phases[a]

Positional isomer	*m*	*n*	SE-30 stationary phase			SP-2330 stationary phase		
			Thia	Selena	Tellura	Thia	Selena	Tellura
2–	0	9	13.4	14.4	15.2	14.6	16.4	16.9
3–	1	8	13.6	14.6	15.3	16.5	18.4	18.6
4–	2	7	13.7	14.6	15.5	16.7	18.5	18.8
5–	3	6	13.8	14.6	15.5	17.0	18.8	19.1
6–	4	5	13.8	14.8	15.6	17.5	19.3	19.1
7–	5	4	13.8	14.8	15.8	17.5	19.4	19.3
8–	6	3	13.8	14.8	15.8	17.6	19.5	19.3
9–	7	2	13.9	15.0	15.9	17.7	19.7	19.5
10–	8	1	14.0	15.1	16.1	17.9	20.0	19.8
11–	9	0	14.1	15.4	16.3	18.3	20.4	20.3

[a]$CH_3(CH_2)_n$-*X*-$(CH_2)_m COOCH_3$ where $n + m = 9$ and *X* = S, Se, or Te.

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ARTICLE

Low Fat–Monounsaturated Rich Diets Containing High-Oleic Peanuts Improve Serum Lipoprotein Profiles[1]

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ABSTRACT: Postmenopausal hypercholesterolemic women are at risk for cardiovascular disease and are encouraged to follow low-fat (LF) (≤30% energy) diets. However, these diets may have undesirable effects on high density lipoprotein cholesterol (HDL-C), apolipoprotein A-I (apo A-I) and triglycerides, whereas diets high in monounsaturated fats do not. Twenty postmenopausal hypercholesterolemic women previously consuming high-fat diets (34% energy) were placed on a low fat–monounsaturated rich diet (LFMR: 26%, 14% energy, respectively) for 6 mon. Sixteen women already eating LF diets (24% energy) were also followed to monitor variations in serum lipids due to seasonal variations. Twenty-five women successfully completed the study (LFMR = 12, LF = 13). Serum cholesterol decreased 10% (264 to 238 mg/dL, $P \leq 0.01$) and low density lipoprotein cholesterol (LDL-C) decreased 12% (182 to 161 mg/dL, $P \leq 0.01$) in the LFMR group, but did not change in the LF group. The reduction in serum cholesterol in the LFMR group was greater than estimated by predictive formulas. Serum triglycerides and apo A-I did not change in the LFMR group. A modest decrease in HDL-C, HDL_3-C, and apolipoprotein B (apo B) occurred in both groups, but only the LFMR group showed a trend toward beneficial changes in LDL-C/HDL-C and apo A-I/apo B ratios. Overall, the LFMR diet was well tolerated and resulted in an improved serum lipid and apolipoprotein profile. *Lipids 32*, 687–695 (1997).

Coronary heart disease (CHD) is the leading cause of death for women over the age of 40 yr (1). The incidence of CHD in women is low prior to menopause; however, after the onset of menopause, it increases to approximately that of men. In the United States, the mean total serum cholesterol for women prior to age 45 is 200–215 mg/dL (5.12–5.56 mmol/L) but rises to 240–260 mg/dL (6.20–6.72 mmol/L) between the ages of 45–54 (2). Currently, the initial treatment for hypercholesterolemia is the Step 1 diet (≤30% energy from fat, ≤10% energy from saturated fat, ≤300 mg cholesterol/d), which generally results in significant reductions in CHD risk factors such as serum cholesterol, low density cholesterol (LDL-C), and apolipoprotein B (apo B). However, low-fat (LF) diets often reduce high density lipoprotein cholesterol (HDL-C) and apolipoprotein A-I (apo A-I), and elevate serum triglycerides (3–5). These changes in the serum profile may be particularly detrimental for women, since serum levels of HDL-C, triglycerides, and the ratio of apo A-I/apo B may be more important in defining the risk of heart disease in this population (6–8). In contrast, diets low in saturated fatty acids (SFA) but high in monounsaturated fatty acids (MUFA) have resulted in reductions in serum cholesterol and LDL-C concentrations without adversely affecting HDL-C, apo A-I, or triglycerides (9–12). Most of the high MUFA diets studied to date, however, are also high in overall fat content (35–40% energy), and are therefore not recommended for treating hypercholesterolemia. Short-term (3–4 wk) controlled studies using diets moderate in fat but relatively high in MUFA have lowered serum cholesterol and LDL-C, but have had inconsistent effects on HDL-C and apo A-I in hypercholesterolemic men and women (13–16). At the time the present study was initiated, it could not be assumed that similar effects would be achieved in a free-living population. Furthermore, it was unclear if postmenopausal women are responsive to dietary treatment, or if diet modification may adversely alter the serum lipid profile. No long-term intervention study using a LF diet rich in MUFA has been conducted exclusively on postmenopausal women.

Most experimental diets have used olive oil, canola oil, or high-oleic sunflower oil as the source of MUFA, but it is also feasible to use nuts containing high amounts of oleic acid (17–19). A high-oleic acid peanut cultivar has been developed at the University of Florida in which 76–80% of the lipid content is MUFA (20), approximately 60–70% more oleic acid than most peanut cultivars. There are several advantages of using the high-oleic peanut as the MUFA source. The low lysine/arginine ratio of peanuts may promote serum cholesterol reduction and other physiological changes which

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

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Abbreviations: apo, apolipoprotein; BMI, body mass index; CHD, coronary heart disease; HDL, high density lipoprotein; HDL-C, high density lipoprotein cholesterol; LDL, low density lipoprotein; LDL-C, low density lipoprotein cholesterol; LF, low fat; LFMR, low fat–monounsaturated rich; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; UF, unsaturated fat.

protect against atherosclerosis (21,22). Frequent consumption of nuts, including peanuts, has been associated with a lower incidence of CHD (23). Peanuts are widely accepted, versatile, and convenient to eat, which may enhance long-term compliance to this LF diet in a free-living population.

The purpose of this study was to determine if a LF diet rich in MUFA from high-oleic peanuts would result in an improved serum lipid and apolipoprotein profile in free-living postmenopausal hypercholesterolemic women.

SUBJECTS AND METHODS

Design. This study was designed to be a 6-mon diet intervention using free-living hypercholesterolemic postmenopausal women who switched from a typical American diet to a LF–monounsaturated rich diet (LFMR). Results were compared to a cohort. Serum lipids and apolipoproteins, body composition, health status, physical activity, and diet composition were measured before initiation of the study and after 6 mon of the diets. The University of Florida Institutional Review Board approved the screening and experimental protocol for this study.

Subjects. Healthy postmenopausal women (50–65 yr of age) with elevated serum cholesterol (220–300 mg/dL; 5.68–7.76 mmol/L) were recruited from the region. To minimize the probability of genetic hyperlipidemia or secondary hyperlipidemias, prospective subjects were eliminated for the following reasons: family history of premature death from heart disease coupled with a serum cholesterol >300 mg/dL (7.76 mmol/L); obesity; serum triglyceride >200 mg/dL; endocrine disorders; hypertension (>140/90 mm Hg); cigarette smoking; consumption of >3 alcoholic beverages per day; hormone replacement therapy; and taking medications that alter blood lipids. Over 350 potential participants were interviewed, and 36 met the criteria to participate in the study. Subjects were unpaid volunteers who gave informed consent to participate in the study. All vitamin/mineral supplements were discontinued 3 mon prior to initiation of the study.

Twenty women who habitually consumed a typical American diet (34% fat, 11% SFA) were assigned to the LFMR diet. It was desirable to have a cohort to control for the seasonal variation in serum lipids and lipoproteins, which has been reported in some long-term studies (24,25). However, having a cohort continue on a high-fat diet posed an ethical dilemma; allowing hypercholesterolemic women to continue eating high-fat diets during a long-term study would continue placing the group at risk for CHD and would be comparable to denying necessary medical treatment. As an alternative, the cohort was constituted from the other 16 hypercholesterolemic women; they were already following self-selected LF diets (LF:20–30% fat, <10% SFA, <300 mg cholesterol). The LF cohort group was instructed to continue following their LF diet. Eight women from the LFMR group and three women from the LF group were unable to complete the study due to injury, illness, schedule conflicts, or noncompliance with the study protocol. The results of the subjects who successfully completed the study are reported (LF = 13, LFMR = 12).

Diets. All participants were taught how to weigh, measure, and record all foods and beverages consumed. Participants completed Seven-Day Diet Records prior to the initiation of the study, and the energy level for weight maintenance was determined by computer analysis (Professional Dietitian, Wellsource, Clackamas, OR) of each subject's baseline diet record. This database contains the nutrient content of approximately 3000 foods and beverages. Information was obtained from the United States Department of Agriculture (USDA) Handbook 8 series (26–33), USDA Handbook 456 (34), Pennington and Church (35), and manufacturers. The nutrient database was expanded by D.J. O'Byrne to include specific processed foods and information on the dietary fiber content of frequently eaten foods (26–33, 36–38, manufacturers' information).

A registered dietitian instructed all participants on their diet protocols. Because subjects were free-living, Daily Eating Plans were given to provide standard guidelines for food selections that would conform to the energy requirements and diet assignment. The LF and LFMR diets had energy distributions of <30% fat, 50–60% carbohydrate, and 15–20% protein. Dietary SFA was restricted to <10% energy, and dietary cholesterol was limited to <300 mg/d. Monounsaturated fat constituted between 50–60% of the fat in the LFMR diet; the main source was high-oleic peanuts. Subjects assigned to the LFMR diet received prepackaged daily rations of dry-roasted high-oleic peanuts. Depending upon energy requirements, LFMR subjects consumed 35–68 g peanuts per day. The peanuts replaced an average of 1 oz (28 g) of cooked lean meat and 3–4 servings of oil or margarine (equivalent of 15–20 g of fat) in the diet.

All participants received educational materials and monthly nutrition/cooking classes to ensure compliance with assigned diets. Subjects were asked to record their food and beverage intake for seven consecutive days each month of the study and to maintain their weight and activity level. Compliance was determined by monthly telephone conversations and evaluation of subject's body weight and Seven-Day Diet Records. A detailed nutrient analysis of each subject's initial and final Seven-Day Diet Record was performed by computer.

Physical measurements. The following measurements were made on each subject prior to the beginning of the diet period and at the end of the 6-mon dietary period: weight, body mass index (BMI) (39), and percentage body fat based on the skinfold thickness of seven anatomical sites (40). Subjects completed a questionnaire on physical activity prior to initiation of the diet and after completion of the study. Weight was measured monthly.

Laboratory methods. Blood samples were drawn after a 12-h fast. Subjects refrained from vigorous exercise and alcohol consumption for 24 h prior to donating blood. Three blood samples were obtained from each subject at 1-wk intervals prior to the beginning of the study and again after 6 mon of dietary treatment. Serum lipid concentrations were measured in triplicate from each sample using microplate methods (41) with reagents from enzymatic kits (Stanbio Enzymatic Triglycerides Procedure No. 2000, Stanbio Enzymatic Cholesterol Procedure No. 1010, and Stanbio Enzymatic HDL-Cho-

lesterol Procedure No. 0599; Stanbio, Houston, TX). Reference sera were included in each assay (Fischer Diagnostics, Orangeburg, NJ). Serum HDL_3 was isolated from total HDL by precipitation with 1.5 M $MgCl_2$ in aqueous 1% dextran sulfate as described by Patsch *et al.* (42). The concentration of HDL_3-C was determined in a similar manner as HDL-C (Stanbio Enzymatic HDL-Cholesterol Procedure No. 0599), except that the dilution correction factor used to take into account the precipitating reagents was 1.21 rather than 1.1. Serum HDL_2-C was calculated by subtracting the concentration of HDL_3-C from HDL-C. The concentration of LDL-C was calculated by the Friedewald formula (43). Freshly isolated serum from each subject was flushed with nitrogen and stored at −70°F (−57°C) until used for apolipoprotein analysis at the end of the study. The serum concentrations of apo A-I, A-II, and B were measured in triplicate from one blood sample taken from each subject prior to initiation of the study and after 6 mon of following the diet. Serum concentrations were determined by a microplate method (41) using turbidimetric test kit reagents and standards (Boehringer Mannheim Biochemica, Indianapolis, IN).

Statistical analysis. Preliminary measurements of serum cholesterol were made on volunteers for the study. Forty-three of 75 prospective subjects had mean serum cholesterol concentrations between 5.68–7.76 mmol/L. Of this group, the mean concentration was 6.55 mmol/L and the sample variance was 10.04 mmol/L. This study was designed to detect a change in serum cholesterol of at least 10% of the sample mean (approximately 0.655 mmol/L). The sample size for each group was calculated using the following formula: $n = 2\sigma^2 [(z_\alpha + z_\beta)^2/\Delta^2]$ (44). In this formula, n = number of subjects in each group; σ^2 = estimated sample variance; $z_\alpha = 1.64$, which corresponds to a one-sided test with $\alpha = 0.05$; $z_\beta = 1.28$, which corresponds with the power = 0.9; and Δ = anticipated change in serum cholesterol. Using the above formula, it was determined that at least 10 subjects were needed in each group in order to have a sufficient sample size. Results are expressed as the mean ± standard deviation. Because subjects were not randomly assigned to the diet groups, Levene's Test for Variance Homogeneity (45) was performed to determine if the sample distribution was similar in both groups. The significance level was set at $P = 0.05$. The Levene's Test showed significant variance for several dietary factors, and log transformations were performed on these data. For variables that had similar sample distribution, an analysis of variance with repeated measures was used to test for significant differences "within" and "between" the two groups ($P = 0.05$). If a significant interaction was detected, a follow-up *t*-test was conducted. A nonparametric test (46) was used on variables in which data transformation did not successfully reduce the sample variance. Analyses were performed using the Statistical Analysis System software (SAS Institute, Inc., Cary, NC).

Correlations between the mean change in variables selected *a priori* were performed to assess the impact of specific dietary components on serum lipids and apolipoproteins and to identify potential confounding factors. These statistical analyses were performed using Microsoft Excel® 4.0 (Microsoft Corporation, Redmond, WA) statistical program.

RESULTS

Diets. Subjects following the LFMR diet tolerated the daily ration of peanuts without difficulties. Initially, some subjects complained of gastrointestinal bloating and cramping, but this subsided with increased fluid intake and consumption of peanuts throughout the day rather than at one time.

The LF and LFMR diets were designed to maintain weight and meet the subjects' nutritional requirements. Dietary energy level was intended to be the same as baseline, but subjects in both groups reduced energy intake (Time Effect, $P \leq 0.01$) by approximately 812–1011 kJ (Table 1). An examination of diet records showed that subjects in each group

TABLE 1
Calculated Daily Energy and Nutrient Intake at Baseline and While Subjects Followed the Low-Fat (LF) or Low Fat–Monounsaturated Rich (LFMR) Diets[a]

	LF (n = 13)		LFMR (n = 12)	
	Before	After	Before	After
Energy (kJ/d)[b,**]	7063 ± 1594	6054 ± 1351	7736 ± 1749	6925 ± 1381
%Fat[c]	23 ± 6	17 ± 4**	34 ± 5	26 ± 3**
%SFA[c]	7 ± 3	5 ± 1**	11 ± 2	5 ± 1**
%MUFA[d,**]	7 ± 2[a]	6 ± 2[a]	11 ± 2[b]	14 ± 1[c]
%PUFA[d,*]	4 ± 2[a]	2 ± 1[b]	6 ± 2[c]	2 ± 1[b]
%Protein[b,**;e,**]	19 ± 3	22 ± 3	15 ± 2	20 ± 2
%Carbohydrate[b,**;e,**]	60 ± 7	63 ± 5	50 ± 7	55 ± 5
%Alcohol	0 ± 1	1 ± 3	2 ± 4	1 ± 3
Cholesterol (mg)[b,**]	182 ± 87	129 ± 36	222 ± 68	130 ± 52
Fiber (g)[d,**]	14 ± 5[a,b]	17 ± 4[b]	11 ± 4[a]	22 ± 8[c]

[a]Mean ± standard deviation, calculated from 7 d of recorded food and beverage intake prior to initiation of the study and after 6 mon of following the assigned diets. *$P \leq 0.05$, **$P \leq 0.01$.
[b]Time effect; analysis of variance (ANOVA).
[c]Data for each group were analyzed by nonparametric tests. The LF and LFMR groups could not be compared.
[d]Significant ANOVA interaction between diet and time; group means that are statistically significant have different letter superscripts.
[e]Group effect, ANOVA. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

TABLE 2
Calculated Nutrient Ratios at Baseline and While Subjects Followed the LF or LFMR Diets[a]

	LF (n = 13)		LFMR (n = 12)	
Ratios	Before	After	Before	After
SFA/MUFA[b]	0.95 ± 0.20	0.90 ± 0.30	0.98 ± 0.20	0.39 ± 0.06**
SFA/UFA[b]	0.62 ± 0.16	0.61 ± 0.19	0.64 ± 0.12	0.33 ± 0.04**
PUFA/MUFA[c,**]	0.57 ± 0.16[a]	0.45 ± 0.12[a]	0.54 ± 0.22[a]	0.18 ± 0.04[b]
SFA/fat[c,**]	0.29 ± 0.06[a]	0.28 ± 0.05[a]	0.32 ± 0.04[a]	0.21 ± 0.02[b]
MUFA/fat[c,**]	0.31 ± 0.04[a]	0.34 ± 0.10[a]	0.33 ± 0.05[a]	0.54 ± 0.03[b]
PUFA/fat[c,d,**]	0.18 ± 0.05[a]	0.15 ± 0.02[a]	0.17 ± 0.04[a]	0.10 ± 0.02[b]
Lysine/arginine[c,**]	1.42 ± 0.12[a]	1.48 ± 0.09[a]	1.48 ± 0.34[a]	0.97 ± 0.17[b]

[a]Mean ± standard deviation, calculated from 7 d of recorded food and beverage prior to initiation of the study and after 6 mon of following the assigned diets. **$P \leq 0.01$.
[b]Data for each group were analyzed by nonparametric tests. The LF and LFMR groups could not be compared.
[c]Significant ANOVA interaction between diet and time; group means that are statistically significant have different letter superscripts.
[d]Log transformation performed prior to ANOVA. Nontransformed data are shown. UFA = unsaturated fatty acids; see Table 1 for other abbreviations.

began selecting specific food items that were lower in fat and energy than foods eaten at baseline. This may have accounted for the slight decrease in energy intake. The final diets of all subjects provided at least 75% of the Recommended Dietary Allowance for major vitamins and minerals (data not shown). The LFMR group altered their diet to meet the experimental guidelines by increasing %MUFA ($P \leq 0.01$) and lowering %fat ($P \leq 0.01$), %SFA ($P \leq 0.01$), % polyunsaturated fatty acids (PUFA) ($P \leq 0.05$), and cholesterol ($P \leq 0.01$). Subjects in the LF group were already following LF diets at the beginning of the study; thus their baseline diets were significantly different from the LFMR group. The LF group participated in nutrition and cooking classes with the LFMR subjects, which may have inadvertently influenced LF subjects to further restrict %fat ($P \leq 0.01$), %SFA ($P \leq 0.01$), %PUFA ($P \leq 0.05$), and dietary cholesterol ($P \leq 0.01$). Because dietary fat was reduced in both groups, the proportion of energy derived from protein and carbohydrates increased (time effects, $P \leq 0.01$). However, the LF group consistently consumed a diet higher in percentage protein and percentage carbohydrate (Group Effects, $P \leq 0.01$). Dietary fiber increased in both groups, but this was only significant for the LFMR group ($P \leq 0.01$).

The most notable difference between the LF and LFMR diets was the MUFA and fatty acid composition of the diet. Subjects in the LFMR group increased dietary MUFA and ate more than twice the proportion of energy as MUFA compared to the LF group. Fatty acid ratios were similar in the LF and LFMR groups at baseline and did not change in the LF group (Table 2). In contrast, the following dietary fatty acid ratios were reduced ($P \leq 0.01$) in the LFMR group: SFA/MUFA, SFA/UFA, PUFA/MUFA, SFA/fat, and PUFA/fat. The MUFA/fat ratio increased significantly ($P \leq 0.01$) so that LFMR subjects were consuming more than half of all dietary fat as MUFA.

Baseline diets contained similar ratios of lysine to arginine (Table 2). Even though the LF group ate more protein, the ratio of lysine to arginine did not change. Because peanuts are high in arginine, the LFMR diet resulted in a significantly lower lysine/arginine ratio ($P \leq 0.01$).

Physical measurements. Figure 1 shows the monthly weight of subjects. There was a trend toward weight loss during the last month of the study, but the LFMR group experienced a gradual and continuous trend toward weight loss during the entire study. After following the LFMR diet for 6 mon, subjects lost approximately 3 kg ($P \leq 0.01$) while the LF group maintained their weight (Table 3). Four of the LFMR subjects experienced weight loss in excess of 3.6 kg. Reported physical activity did not change in either group (data not shown). The subjects who lost the most weight expressed difficulty in consuming all the required food. When their data were eliminated from the group mean, the mean weight loss for the LFMR group was only 2.1 kg. As a consequence of the weight loss, BMI decreased ($P \leq 0.01$) and body fat was slightly lower in the LFMR group.

Serum lipid and apolipoprotein profile. The baseline serum lipid concentrations for cholesterol, LDL-C, and cholesterol ratios (Table 4) were higher in the LFMR group compared to the LF group. After 6 mon of following the LFMR diet, serum cholesterol and LDL-C fell by approximately 10% and 12%,

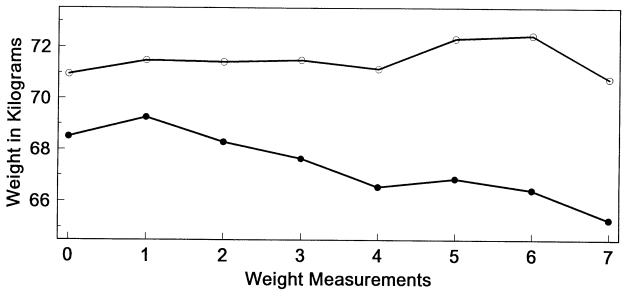

FIG. 1. Mean weight in kilograms, of the postmenopausal hypercholesterolemic women in the low-fat (LF) -o- (n = 13) and low fat–monounsaturated rich (LFMR) -●- (n = 12) group at baseline (0), and at seven time points during the 6-mon dietary period.

TABLE 3
Characteristics of Subjects Following the LF Diet and the LFMR Diet[a]

Physical measurement	LF (n = 13)		LFMR (n = 12)	
	Before	After	Before	After
Weight (kg)[b,**]	71.0 ± 10.5[a]	70.8 ± 11.0[a]	68.6 ± 6.2[b]	65.3 ± 6.5[c]
% Body fat[c,*]	34.5 ± 3.6	34.0 ± 5.5	34.6 ± 3.6	31.5 ± 3.5
Body mass index[b,**]	26.4 ± 3.3[a]	26.3 ± 3.6[a]	26.2 ± 3.8[a]	24.9 ± 3.8[b]

[a]Mean ± standard deviation, obtained prior to initiation of the study and after 6 mon of following the assigned diets. *$P \leq 0.05$, **$P \leq 0.01$.
[b]Significant ANOVA interaction between diet and time; group means that are statistically significant have different letter superscripts.
[c]Time effect. See Table 1 for abbreviations.

TABLE 4
Concentrations of Serum Lipids and Lipid Ratios in Subjects Before and After Following the LF and LFMR Diets (for 6 mon)[a]

Serum lipids	LF (n = 13)		LFMR (n = 12)	
	Before	After	Before	After
	mg/dL	mg/dL	mg/dL	mg/dL
Triglyceride	120 ± 43	134 ± 42	157 ± 67	156 ± 72
Cholesterol[b,**]	247 ± 19[a]	241 ± 22[a]	264 ± 25[b]	238 ± 24[c]
LDL cholesterol[b,**]	170 ± 17[a]	166 ± 21[a]	182 ± 20[b]	161 ± 19[c]
HDL cholesterol[c,**]	52 ± 11	48 ± 10	50 ± 8	46 ± 9
HDL_2 cholesterol	11 ± 10	12 ± 8	8 ± 7	10 ± 7
HDL_3 cholesterol[c,**]	41 ± 4	36 ± 4	43 ± 4	36 ± 4
Total-C/HDL-C[b,*]	4.92 ± 0.97[a]	5.20 ± 1.10[b]	5.37 ± 0.77[b]	5.32 ± 0.94[b]
LDL-C/HDL-C[b,*]	3.42 ± 0.78[a]	3.61 ± 0.91[a,b]	3.69 ± 0.44[b]	3.58 ± 0.58[b]

[a]Mean ± standard deviation. The mean concentration is the average value of three fasting serum samples; each sample was measured in triplicate. Lipid ratios are calculated from mean serum lipid concentrations.
*$P \leq 0.05$, **$P \leq 0.01$.
[b]Significant ANOVA interaction between diet and time; group means that are statistically significant have different letter superscripts.
[c]Time effect, ANOVA. Total-C/HDL-C = ratio of serum total cholesterol to serum high density lipoprotein cholesterol. LDL-C/HDL-C = ratio of serum low density lipoprotein cholesterol to serum high density lipoprotein cholesterol. See Table 1 for other abbreviations.

respectively ($P \leq 0.01$), and triglyceride levels were stable. Serum cholesterol and LDL-C did not change in the LF group, but triglycerides rose by 14% ($P = 0.056$). Both groups experienced a 4 mg/dL (0.10 mmol/L) decrease in HDL-C (time effect, $P \leq 0.01$). This modest reduction was due to changes in the cholesterol content of the HDL_3 subfraction (time effect, $P \leq 0.01$). The ratios of total cholesterol/HDL-C and LDL-C/HDL-C increased in the LF group ($P \leq 0.05$, $P = 0.058$, respectively) but were lowered by 0.05–0.11 in the LFMR group.

A reduction in serum apo A-I (time effect, $P \leq 0.01$) and apo B concentrations (time effect, $P \leq 0.05$) occurred in both groups (Table 5). Although results from the analysis of variance indicated an effect of time on the concentration of apo A-I, this was primarily due to the 13% decrease in serum values in the LF group ($P \leq 0.01$). In contrast, the LFMR group experienced a small but nonsignificant reduction in apo A-I concentration. Apo B concentrations were lowered to a similar extent in both groups, and the final mean values were within the normal range of 0.60–1.00 g/L. As a result of the changes in apo A-I and apo B concentrations, the ratio of apo A-I/apo B increased slightly in the LFMR group but decreased in the LF group.

DISCUSSION

The LFMR diet resulted in significant reductions in serum cholesterol and LDL-C. These results are in line with short-term studies in which controlled LF diets relatively high in MUFA reduced serum cholesterol by 7–15% and LDL–C by 11–16% in hyperlipidemic males and females (13–15). A seasonal effect on these variables was discounted since the LF group had no reduction in serum cholesterol or LDL-C. Our study demonstrates that free-living postmenopausal women are responsive to diet therapy, and that the LFMR diets elicit reductions in serum cholesterol levels similar to more controlled diets.

The hypocholesterolemic effects of the LFMR diet were underestimated by established predictive equations (47–50). These equations estimated a 3.4–4.0% decrease in serum cholesterol compared to the 10% reduction observed. Yet, when applied to the LF group, these formulas accurately predicted

TABLE 5
Concentrations of Serum Apolipoproteins in Subjects Before and After Following the LF and LFMR Diets (for 6 mon)[a]

	LF (n = 13)		LFMR (n = 12)	
	Before	After	Before	After
Apo A-I (g/L)[b,**]	1.90 ± 0.28	1.62 ± 0.28	1.70 ± 0.24	1.60 ± 0.37
Apo A-II (g/L)	0.48 ± 0.08	0.46 ± 0.12	0.43 ± 0.11	0.42 ± 0.09
Apo B (g/L)[b,*]	1.09 ± 0.18	0.98 ± 0.25	1.08 ± 0.16	0.99 ± 0.17
Apo A-I/Apo B	1.81 ± 0.53	1.75 ± 0.50	1.59 ± 0.26	1.61 ± 0.24

[a]Mean ± standard deviation. The mean concentration of each apolipoprotein was determined on a fasting serum sample and measured in triplicate. *$P \leq 0.05$, **$P \leq 0.01$.
[b]Time effect, ANOVA. Apo = apolipoprotein. See Table 1 for other abbreviations.

the reduction in serum cholesterol (1.8% vs. 1.4–1.5%, respectively). It is important to point out that the predictive equations account only for the influence of dietary cholesterol, SFA, and PUFA on serum cholesterol. Other components of the LFMR diet may have had an additional impact on serum cholesterol. Significant correlations were detected in the LFMR group between the change in serum cholesterol and changes in the amount of carbohydrate (r = –0.842, $P < 0.05$), % fat (r = 0.619, $P < 0.05$), amount of protein (r = –0.603, $P < 0.05$), and MUFA/fat (r = –0.572, P = 0.055). These correlations are largely consistent with the known cholesterol-lowering effects of a LF diet and of replacing SFA with MUFA. Additional factors such as the arginine, phytosterol, and fiber content of the LFMR diet were also analyzed to determine their possible influence on serum cholesterol. The low lysine/arginine ratio of the LFMR diet was expected to promote cholesterol-lowering, but the change in this ratio was not correlated with changes in serum cholesterol (r = –0.195) or LDL-cholesterol (r = –0.085). Phytosterols have been shown to lower serum cholesterol levels, presumably by inhibiting cholesterol absorption (51,52). The phytosterol content of foods has not been thoroughly studied; therefore, the nutrient database used in this study does not contain sufficient information to provide more than a crude approximation of the amount of phytosterols in subjects' diets. Based on information available, the phytosterol content of the LFMR diet appeared to increase (71 ± 42 vs. 147 ± 29 mg/d, $P < 0.001$), whereas the amount in the LF diet did not change (55 ± 40 vs. 41 ± 16 mg/d). It is doubtful whether the small increase in phytosterols influenced serum levels in the LFMR group, since other studies have shown the addition of 500–3400 mg/d of phytosterols is required to elicit significant reductions in serum cholesterol and LDL-C (51,52). Furthermore, no correlation was found between the change in dietary phytosterol content and the change in serum total cholesterol (r = –0.251) and LDL-C (r = 0.199) in the LFMR group. The fiber content in the LFMR diet also increased from 11 to 22 g/d. Peanuts contributed 2.4–4.5 g of dietary fiber to the LFMR diet, but <0.2 g was soluble fiber (38). Dietary fiber has been found to lower serum cholesterol when consumed in excess of 49 g/d as part of a LF diet, or when diets have been supplemented with 12–45 g of purified soluble fiber (53,54). The modest increase in dietary fiber in the LFMR diet did not correlate with changes in serum cholesterol (r = –0.365).

Participants in the study were free-living and as a consequence, food intake could not be strictly controlled. Both groups lowered energy intake below their baseline diets. The reduced energy intake by the LFMR group might account for their weight loss. It is interesting to note that both groups had similar reductions in energy intake, but only the LFMR group lost weight. Serum lipids can be influenced by weight loss, and a significant correlation was seen between changes in weight and cholesterol in the pooled data (r = 0.483). When the LFMR group was analyzed separately, no significant correlations were found between the changes in serum cholesterol and changes in weight (r = 0.545), even though final weights ranged from +2.0 to −5.5 kg of baseline. The influence of weight on serum cholesterol should not be dismissed entirely since the correlation was approaching statistical significance, but of more clinical relevance is the lack of significant correlation between changes in weight and serum LDL-C in the LFMR group (r = 0.397). Nevertheless, it is possible that an interaction between the LFMR diet and weight loss elicited the hypocholesterolemic effects. A linear regression was performed on the pooled data of both groups, and no interaction was found between the LFMR diet and weight change (P = 0.839). The main effect of the LFMR diet on changes in serum total cholesterol was significant (P = 0.045), but the effect of weight change was not (P = 0.185). With the relatively small sample size in this study, it is difficult to separate out the potentially confounding effects of modest weight loss, and it is possible that changes in weight had a slight effect.

Long-term consumption of the LFMR diet resulted in apo B levels within the normal range. Of interest in our experiment was the trend toward lower apo B values even in the LF group. Since the LFMR and LF groups reduced dietary fat, SFA, and cholesterol, the influence of diet modifications probably explains the similar changes in apo B, but seasonal variations cannot be ruled out.

The high MUFA content of the LFMR diet was expected to prevent an increase in serum triglycerides and a decrease in HDL-C and apo A-I, when total dietary fat and saturated fat were reduced. Serum triglycerides and apo A-I did remain stable in the LFMR group, but were adversely affected in the LF group. This suggests that the MUFA content of the LFMR diet attenuated the changes in serum triglycerides and apo A-I that are usually associated with LF-high carbohydrate diets.

This was also evidenced by the slight increase in apo A-I/apo B ratios in the LFMR group, in contrast to the downward trend in the LF group. The modest reduction in HDL-C after the LFMR diet is consistent with that reported for short-term controlled studies of LF diets containing 14–16% MUFA (13–16). It was expected that subjects would consume 16–19% MUFA, but the calculated content of the LFMR diet was only 14%. This was due to inconsistent consumption of canola and olive oils, and overall restriction of dietary fat. One study has shown that a LF diet containing approximately 17% MUFA from olive oil prevented reductions in HDL-C (15). If the LFMR subjects had consumed a higher proportion of MUFA, HDL-C levels might have been spared. Although a reduction in HDL-C is not desirable, this change was a reflection of the lower cholesterol concentration in the HDL_3 subfraction, rather than HDL_2. Epidemiological and clinical data indicate that the inverse relationship seen between HDL-C and premature atherosclerosis is stronger for HDL_2-C than for HDL_3-C (55,56). Because the concentrations of HDL_2-C and apo A-I did not change and the ratio of LDL-C/HDL-C showed a beneficial downward trend, the protective role of HDL was probably not compromised by the LFMR diet.

In summary, the LFMR diet was well tolerated and the high-oleic peanuts were easily incorporated into each subject's daily diet. This diet resulted in significant reductions in serum cholesterol and LDL-C and a trend toward improved lipid and apolipoprotein ratios. The hypocholesterolemic effects of the LFMR diet were greater than expected by predictive equations, which suggests that the use of high-oleic peanuts as the primary source of MUFA may be more advantageous than typical high-oleic oils or other vegetable oils. Furthermore, serum triglycerides, HDL_2-C, and apo A-I were not adversely affected by the LFMR diet. This study documents that free-living postmenopausal women can achieve improved serum lipid and apolipoprotein levels on self-selected LF diets high in MUFA.

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Incorporation of n-3 Fatty Acids into Plasma Lipid Fractions, and Erythrocyte Membranes and Platelets During Dietary Supplementation with Fish, Fish Oil, and Docosahexaenoic Acid-Rich Oil Among Healthy Young Men

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ABSTRACT: The effects of n-3 fatty acid supplementation in the form of fresh fish, fish oil, and docosahexaenoic acid (DHA) oil on the fatty acid composition of plasma lipid fractions, and platelets and erythrocyte membranes of young healthy male students were examined. Altogether 59 subjects (aged 19–32 yr, body mass index 16.8–31.3 kg/m^2) were randomized into the following diet groups: (i) control group; (ii) fish diet group eating fish meals five times per week [0.38 ± 0.04 g eicosapentaenoic acid (EPA) and 0.67 ± 0.09 g DHA per day]; (iii) DHA oil group taking algae-derived DHA oil capsules (1.68 g/d DHA in triglyceride form); and (iv) fish oil group (1.33 g EPA and 0.95 g DHA/d as free fatty acids) for 14 wk. The fatty acid composition of plasma lipids, platelets, and erythrocyte membranes was analyzed by gas chromatography. The subjects kept 4-d food records four times during the study to estimate the intake of nutrients. In the fish diet, in DHA oil, and in fish oil groups, the amounts of n-3 fatty acids increased and those of n-6 fatty acids decreased significantly in plasma lipid fractions and in platelets and erythrocyte membranes. A positive relationship was shown between the total n-3 polyunsaturated fatty acids (PUFA) and EPA and DHA intake and the increase in total n-3 PUFA and EPA and DHA in all lipid fractions analyzed. DHA was preferentially incorporated into phospholipid (PL) and triglyceride (TG) and there was very little uptake in cholesterol ester (CE), while EPA was preferentially incorporated into PL and CE. The proportion of EPA in plasma lipids and platelets and erythrocyte membranes increased also by DHA supplementation, and the proportion of linoleic acid increased in platelets and erythrocyte membranes in the DHA oil group as well. These results suggest retroconversion of DHA to EPA and that DHA also interferes with linoleic acid metabolism.

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Recent studies have demonstrated that increased intake of polyunsaturated fatty acids (PUFA) of the n-3 series, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), may have a favorable effect on serum lipids (1–3), platelet aggregability (4–6), and bleeding time (4) and thus could lead to a reduced risk of atherosclerotic vascular disease and thrombotic complications. Long-term consumption of fish leads to the increased incorporation of n-3 fatty acids into plasma lipids, erythrocytes, and platelets (7,8). After fish oil supplementation, increased content of EPA and DHA has been measured in plasma lipids (6,8–10) and platelet (11,12) and erythrocyte membranes (13,14) with the simultaneous decrease of arachidonic acid and docosatetraenoic acid content, but less is known about the effect of DHA supplementation alone. DHA is a major n-3 fatty acid of most fish, yet most commercial fish oils contain more EPA than DHA (15). The aim of the present study was to examine the long-term effects of the supplementation of three different sources of EPA and DHA on the incorporation of fatty acids in plasma lipids, erythrocyte membranes, and platelets in healthy young men. In particular, interest was focused on the effects of DHA-rich oil (without EPA), which has been shown to have a comparable hypotriglyceridemic effect with fish diet and fish oil supplementation (16).

SUBJECTS AND METHODS

Subjects. Altogether 59 healthy male students aged 19–32 yr participated in this study. Body mass index (BMI) ranged from 16.8 to 31.3 kg/m^2. One subject in the control and one in the fish diet group were excluded because of high plasma triglyceride (TG) levels (>2.5 mmol/L). One subject did not follow the fish diet and was excluded. One subject in the fish oil group was excluded because of low platelet counts in the beginning of the study ($35 \cdot 10^9$/L). Three subjects (one in the control, one in the fish diet, and one in the DHA oil group) missed the analysis of fatty acid composition. Thus, the final analysis included 52 subjects. In addition, erythrocyte sam-

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Abbreviations: BMI, body mass index; CE, cholesterol ester; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PL, phospholipid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TG, triglyceride.

ples from 13 subjects—7 in the control, 1 in the fish diet, 3 in the DHA oil, and 2 in the fish oil groups—and a platelet sample from 1 subject in the control group were discarded because of contamination during sample preparation.

Study design. The present study was part of a study to investigate the long-term effects of moderate amounts of n-3 fatty acids in different forms on plasma lipids and was carried out at the Department of Physiology, University of Kuopio (Kuopio, Finland). Detailed description of the study has been described elsewhere (16). The protocol was approved by the Ethics Committee of the University of Kuopio. The subjects were randomly allocated into one of the four diet groups: (i) control group; (ii) fish diet group; (iii) DHA oil group, and (iv) fish oil group. The subjects started the study stepwise with 2-wk intervals in three groups. The study period was 14 wk. The subjects visited the research unit four times: at baseline and after 4 wk, 9 wk, and 14 wk. Height was measured at baseline only and weight at every visit. Blood samples for laboratory measurements were taken at every visit after 12 h fasting.

Experimental diets and oil supplements. Subjects in the control group were asked to maintain their normal dietary habits and physical activities constant throughout the study. Subjects in the fish diet group consumed their fish meal at the University Restaurant every working day five times per week. The number of fish meals actually eaten was 4.3 ± 0.5 times per week which produced 0.38 g EPA, 0.08 g docosapentaenoic acid, and 0.67 g DHA per day. Fish used for meals were rainbow trout *(Oncorhynchus mykis),* Balting herring *(Clupea harengus),* and vendace *(Coregonus albula)*. Samples of cooked fish meals were taken every day. Meals containing same-fish species were pooled, and the fatty acid compositions of fish meals were analyzed. In the fish diet group, the intake of fish, except at the University Restaurant, was on an average 14 g/d, supplying 0.05 g EPA and 0.15 g DHA per day according to food records. The subjects in the DHA oil group took eight algae-derived DHA oil capsules/d (Martek Biosciences Corporation, Columbia, MD) supplying 210 mg DHA per capsule (1.68 g/d) in TG form. DHA oil capsules did not contain EPA or docosapentaenoic acid. Fish oil supplement (Bio-Marin, Pharma Nord ApS, Vojens, Denmark) provided 166 mg EPA, 19 mg docosapentaenoic acid, and 119 mg DHA per capsule (1.33, 0.15, and 0.95 g/d, respectively) as free fatty acids. Compliance was assessed by asking for the return of the unused capsules. Except for the oil supplement, the subjects in the DHA oil and fish oil groups were asked to maintain their normal food habits. Dietary fish intake in the DHA oil and fish oil groups during the study was 32 g and 26 g, respectively. At baseline, there were no significant differences in the dietary intake of arachidonic acid among the study groups (ranged from 0.14 to 0.22 g/d). Subjects in the fish oil group received 59 mg/d arachidonic acid from fish oil supplement.

Dietary records. Each participant kept a 4-d food record, using household measures, over three weekdays and one weekend day in the beginning of the study, and three times during the study. Food records were checked by a nutritionist. The intake of nutrients was calculated using the Micro-Nutrica dietary analysis program, based on the database of the Finnish Social Insurance Institute (17). In the results on the nutrient intake, data for each group are given at baseline (4 d) and for the whole study period based on the mean of 12-d food records.

Analysis of fatty acid composition of plasma lipids, platelets, and erythrocyte membranes. Blood samples for laboratory analyses were taken into EDTA-containing vacuum tubes after 12 h fasting. Plasma was separated by centrifugation and stored at −70°C until analyzed. In the analysis of the fatty acid composition of plasma lipids, samples were extracted with chloroform/methanol (2:1, vol/vol) (18), and the lipid fractions were separated by solid-phase extraction with an aminopropyl column (19). For fatty acid analysis of platelets and erythrocyte membranes, the blood samples were rotated at low speed (120 × *g* for 10 min at 4°C) to separate platelet-rich plasma. The platelets were separated by centrifugation at 2,000 × *g* for 15 min at 4°C and then washed with Tris-HCl buffer (pH 7.6, 172 mmol/L). Erythrocytes were washed with the same buffer and hemolyzed in the same diluted buffer (11 mmol/L). Erythrocyte membranes were prepared by centrifugation at 20,000 × *g* for 20 min at 4°C. Both platelet and erythrocyte membrane sediments were suspended in the Tris-HCl buffer (172 mmol/L) and stored at −80°C until analyzed. Lipids were extracted with chloroform/methanol and transmethylated with 14% boron trifluoride in methanol at +100°C for 1 h. Fatty acid methyl esters were analyzed with gas chromatography (HP 5890 Series II; Hewlett-Packard Company, Waldbronn, Germany) equipped with an HP-FFAP capillary column. The molar percentage proportions of fatty acids were calculated.

Statistical methods. Statistical analyses were carried out with the use of SPSS-PC (SPSS, Chicago, IL). The Kruskall-Wallis test was used to compare the between-group differences in the fatty acid composition values at baseline, and within group the changes in the fatty acid composition values from baseline to 14 wk. When the analysis indicated significant change ($P < 0.05$), the Mann-Whitney U-test was used to compare the values of the study groups to those of the control group.

RESULTS

The baseline characteristics of the subjects and the nutrient composition of the diets at baseline are presented in Table 1. Age, height, weight, and BMI were comparable among the study groups. There were no significant differences among the diet groups in the nutrient intake values based on the 4-d food record at baseline compared with those based on the 12-d food record during the study (three 4-d food records combined), except the intakes of total fat and monounsaturated fatty acids (MUFA) in the fish oil group which increased from 30.9 ± 5.7 to 33.7 ± 4.9% and from 11.0 ± 2.3 to 12.5 ± 2.5% of total energy, respectively, $P < 0.01$ in both. In addition, the intake of vitamin D decreased from 5.5 ± 2.6 to 3.9 ± 1.0 μg/d in the control group and increased from 5.1 ± 3.2

TABLE 1
Characteristics of the Study Subjects and the Nutrient Intake at Baseline[a]

	Control $n = 13$	Fish diet $n = 12$	DHA oil $n = 13$	Fish oil $n = 14$
Characteristics				
Age (yr)	23 ± 2	23 ± 2	24 ± 4	23 ± 2
Weight (kg)	75 ± 11	75 ± 12	71 ± 12	78 ± 11
Height (cm)	179 ± 8	182 ± 7	181 ± 6	181 ± 4
BMI (kg/m^2)	23.4 ± 2.1	22.8 ± 3.5	21.5 ± 2.5	23.7 ± 3.3
Nutrient intake				
Energy (MJ/d)	9.9 ± 1.6	10.6 ± 1.7	10.5 ± 2.7	11.3 ± 3.4
Fat (% of energy)	33.0 ± 7.3	36.0 ± 5.4	35.3 ± 7.2	30.9 ± 5.7
SFA (% of energy)	12.9 ± 2.5	14.9 ± 2.4	14.0 ± 3.2	11.8 ± 2.8
MUFA (% of energy)	12.3 ± 3.5	12.6 ± 2.9	12.3 ± 3.1	11.0 ± 2.3
PUFA (% of energy)	5.3 ± 1.8	5.9 ± 1.6	6.4 ± 2.2	5.8 ± 1.9
Protein (% of energy)	15.5 ± 3.1	16.1 ± 1.7	16.0 ± 3.5	16.4 ± 3.7
Carbohydrates (% of energy)	48.0 ± 7.0	43.5 ± 4.6	45.3 ± 7.4	48.0 ± 7.9
Cholesterol (mg/d)	295 ± 134	320 ± 75	312 ± 122	290 ± 87
n-3 Fatty acids from fish diet and supplements				
EPA (g/d)		0.38 ± 0.04	—	1.33
DHA (g/d)		0.67 ± 0.09	1.68	0.95

[a]Values are means ± SD. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; BMI, body mass index.

to 9.2 ± 1.8 μg/d in the fish diet group ($P < 0.05$ and $P < 0.01$, respectively).

Fatty acid composition. The fatty acid composition of plasma TG, cholesterol esters (CE) and phospholipids (PL), and platelets and erythrocyte membranes at baseline are shown in Tables 2–6, as well as the changes in the fatty acid values during 14 wk. The baseline levels of fatty acids in the plasma lipid fractions, erythrocyte membranes, and platelets did not differ among the diet groups.

No significant changes were observed in the fatty acid composition of the control group in either plasma lipids, platelets, or erythrocyte membranes during the study period.

Fatty acid composition of plasma lipids. In the fish diet, in DHA oil and fish oil groups, the amount of n-3 fatty acids increased significantly in TG, CE, and PL, and the amount of n-6 fatty acids decreased significantly in PL when compared with those of the control group. In the fish diet group, the proportions of dihomo-γ-linolenic acid in CE and PL and that of

TABLE 2
Fatty Acid Composition (mol% of total) of Plasma Triglycerides[a]

	Control ($n = 13$)		Fish diet ($n = 12$)		DHA oil ($n = 13$)		Fish oil ($n = 14$)	
Fatty acid	Baseline	Change	Baseline	Change	Baseline	Change	Baseline	Change
SFA	33.4 ± 3.5	0.6 ± 5.2	34.4 ± 4.0	1.9 ± 5.1	35.7 ± 4.3	–0.7 ± 5.3	33.4 ± 4.3	–1.0 ± 6.5
MUFA	45.1 ± 3.0	0.0 ± 6.2	44.6 ± 2.1	–2.8 ± 4.0	44.1 ± 3.6	–3.2 ± 3.3	44.8 ± 3.6	–4.7 ± 6.2
18:1n-9	38.4 ± 3.1	0.2 ± 5.3	38.0 ± 2.3	–2.3 ± 3.9	37.6 ± 2.9	–2.5 ± 2.8	38.3 ± 3.1	–3.8 ± 5.0
PUFA	21.5 ± 4.1	–0.6 ± 6.6	21.0 ± 3.5	0.9 ± 6.1	20.9 ± 4.6	4.0 ± 5.6	21.8 ± 5.9	5.7 ± 6.8
18:2n-6	16.9 ± 3.8	–0.5 ± 6.1	15.6 ± 3.1	–0.9 ± 4.7	15.7 ± 3.7	–0.0 ± 2.7	16.8 ± 4.8	1.3 ± 5.4
18:3n-6	0.4 ± 0.2	–0.1 ± 0.2	0.4 ± 0.2	–0.1 ± 0.2	0.4 ± 0.2	–0.1 ± 0.2	0.4 ± 0.2	–0.1 ± 0.2
18:3n-3	1.6 ± 0.6	0.2 ± 0.5	1.7 ± 0.4	0.0 ± 0.7	1.4 ± 0.3	0.7 ± 0.5	2.0 ± 0.8	0.1 ± 0.9
20:3n-6	0.2 ± 0.1	0.0 ± 0.1	0.2 ± 0.1	–0.0 ± 0.1	0.2 ± 0.1	–0.0 ± 0.1	0.2 ± 0.1	–0.1 ± 0.1
20:4n-6	0.8 ± 0.2	–0.1 ± 0.3	0.9 ± 0.1	–0.0 ± 0.2	0.8 ± 0.3	0.0 ± 0.2	0.8 ± 0.2	0.2 ± 0.2
20:5n-3	0.3 ± 0.2	–0.0 ± 0.2	0.4 ± 0.2	0.6 ± 0.9[b]	0.3 ± 0.2	0.2 ± 0.4[a]	0.4 ± 0.2	1.7 ± 0.6[c]
22:5n-3	0.3 ± 0.1	–0.0 ± 0.1	0.4 ± 0.1	0.1 ± 0.2	0.3 ± 0.1	–0.0 ± 0.2	0.3 ± 0.1	0.5 ± 0.2[c]
22:6n-3	0.9 ± 0.6	–0.1 ± 0.7	1.3 ± 0.6	1.3 ± 1.8[a]	1.0 ± 1.0	3.2 ± 2.7[c]	0.9 ± 0.6	2.0 ± 1.0[c]
∑n-6	18.4 ± 3.8	–0.7 ± 6.2	17.2 ± 3.2	–1.2 ± 4.9	17.2 ± 3.9	–0.1 ± 2.9	18.2 ± 4.9	1.4 ± 5.6
∑n-3	3.1 ± 0.8	0.1 ± 0.9	3.8 ± 0.9	2.1 ± 2.9[a]	3.0 ± 1.3	4.1 ± 3.5[c]	3.5 ± 1.3	4.3 ± 2.0[c]
Quantitative amounts (μg/mL)								
	Baseline	After 14 wk	Baseline	After 14 wk	Baseline	After 14 wk	Baseline	After 14 wk
20:5n-3	2.5 ± 1.2	2.9 ± 2.0	3.3 ± 1.5	7.0 ± 4.0	2.5 ± 1.6	3.5 ± 2.5	3.3 ± 2.9	12.9 ± 5.7[c]
22:6n-3	7.0 ± 3.6	8.4 ± 6.6	11.2 ± 6.9	21.2 ± 10.5	7.8 ± 6.6	28.0 ± 18.7[b]	9.1 ± 7.5	19.8 ± 10.8[b]

[a]At baseline and the changes after 14 wk, and quantitative amounts of eicosapentaenoic and docosahexaenoic acids (μg/mL) at baseline and after 14 wk among the study groups. Values are means ± SD. Superscript letters a, b, c indicate a significant difference from control group, $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively. See Table 1 for abbreviations.

TABLE 3
Fatty Acid Composition (mol% of total) of Plasma Cholesterol Esters[a]

	Control (n = 13)		Fish diet (n = 12)		DHA oil (n = 13)		Fish oil (n = 14)	
Fatty acid	Baseline	Change	Baseline	Change	Baseline	Change	Baseline	Change
SFA	14.9 ± 0.8	1.2 ± 2.3	15.3 ± 1.3	0.7 ± 1.6	15.3 ± 1.8	–0.2 ± 1.3	15.1 ± 1.1	1.8 ± 1.7
MUFA	24.9 ± 3.8	0.6 ± 2.6	23.9 ± 3.1	0.5 ± 4.1	24.4 ± 2.8	–2.0 ± 2.3	23.7 ± 2.8	–1.6 ± 2.6
18:1n-9	20.5 ± 2.4	0.6 ± 2.3	19.7 ± 2.5	0.3 ± 3.0	20.0 ± 2.1	–1.5 ± 1.6	19.3 ± 1.8	–1.2 ± 1.6
PUFA	60.2 ± 4.0	–1.8 ± 4.5	60.8 ± 3.8	–1.2 ± 4.5	60.4 ± 3.9	2.2 ± 3.1	61.3 ± 3.2	–0.2 ± 3.2
18:2n-6	51.4 ± 4.0	–1.0 ± 4.6	52.1 ± 4.0	–2.2 ± 5.1	52.6 ± 3.9	1.9 ± 3.8	53.2 ± 4.1	–4.4 ± 4.3
18:3n-6	0.8 ± 0.3	–0.1 ± 0.2	0.7 ± 0.2	–0.2 ± 0.3	0.7 ± 0.2	–0.3 ± 0.2[a]	0.7 ± 0.4	–0.4 ± 0.2[b]
18:3n-3	0.8 ± 0.3	0.0 ± 0.2	0.8 ± 0.2	0.0 ± 0.2	0.8 ± 0.2	0.1 ± 0.2	0.8 ± 0.2	–0.1 ± 0.2
20:3n-6	0.5 ± 0.1	–0.0 ± 0.1	0.5 ± 0.1	–0.1 ± 0.1[a]	0.4 ± 0.1	–0.1 ± 0.1	0.5 ± 0.2	–0.1 ± 0.1[b]
20:4n-6	4.9 ± 1.3	–0.5 ± 0.6	4.8 ± 1.1	–0.5 ± 0.8	4.3 ± 0.9	–0.5 ± 0.7	4.6 ± 1.2	0.2 ± 0.7[a]
20:5n-3	1.2 ± 0.4	–0.2 ± 0.4	1.3 ± 0.5	1.5 ± 1.0[c]	1.0 ± 0.2	0.5 ± 0.5[c]	1.0 ± 0.3	4.0 ± 1.2[c]
22:6n-3	0.6 ± 0.2	0.0 ± 0.2	0.6 ± 0.2	0.3 ± 0.3[a]	0.5 ± 0.2	0.5 ± 0.3[c]	0.6 ± 0.1	0.5 ± 0.2[c]
∑n-6	57.6 ± 4.1	–1.6 ± 4.6	58.1 ± 3.8	–3.0 ± 5.0	58.0 ± 3.8	1.0 ± 3.4	58.9 ± 3.5	–4.6 ± 4.0
∑n-3	2.6 ± 0.6	–0.2 ± 0.6	2.7 ± 0.6	1.8 ± 1.2[c]	2.3 ± 0.4	1.2 ± 0.9[c]	2.4 ± 0.4	4.4 ± 1.4[c]
Quantitative amounts (μg/mL)								
	Baseline	After 14 wk	Baseline	After 14 wk	Baseline	After 14 wk	Baseline	After 14 wk
20:5n-3	13.0 ± 6.3	9.9 ± 3.6	12.0 ± 5.2	27.1 ± 10.5	9.7 ± 2.8	14.2 ± 6.2	10.5 ± 4.5	45.5 ± 16.1[c]
22:6n-3	6.4 ± 3.2	6.0 ± 2.7	6.0 ± 2.2	9.1 ± 2.8	5.7 ± 2.4	11.2 ± 4.7	6.1 ± 1.8	10.5 ± 3.8[b]

[a]At baseline and the changes after 14 wk, and quantitative amounts of eicosapentaenoic and docosahexaenoic acids (μg/mL) at baseline and after 14 wk among the study groups. Values are means ± SD. Superscript letters a, b, c are significantly different from control group, $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively. See Table 1 for abbreviations.

arachidonic acid in PL decreased significantly. The DHA oil-supplemented subjects had significantly decreased values of γ-linolenic acid in CE and lower values of dihomo-γ-linolenic, arachidonic and docosapentaenoic acids in PL. Compared with the control group, the fish oil-supplemented subjects had significant increases in the proportions of docosapentaenoic acid in TG and PL and arachidonic acid in CE, and decreases in the proportions of γ-linolenic and dihomo-γ-linolenic acids in CE and oleic, linoleic, dihomo-γ-linolenic, and docosatetraenoic acids in PL (Tables 2–4, Fig. 1).

Quantitative amounts of EPA and DHA in plasma lipids. Quantitative amounts of EPA and DHA in plasma lipid fractions are presented in Tables 2–4. The 14-wk fish diet elevated most of the quantitative amounts of EPA in PL and CE and the amount of DHA in PL and TG. After DHA oil supplementation, the quantitative amount of DHA in plasma

TABLE 4
Fatty Acid Composition (mol% of total) of Plasma Phospholipids[a]

	Control (n = 13)		Fish diet (n = 12)		DHA oil (n = 13)		Fish oil (n = 14)	
Fatty acid	Baseline	Change	Baseline	Change	Baseline	Change	Baseline	Change
SFA	48.0 ± 1.2	0.1 ± 1.1	48.6 ± 1.4	–0.4 ± 1.4	47.8 ± 0.9	–0.2 ± 1.5	47.6 ± 1.1	0.7 ± 0.9
MUFA	14.6 ± 1.7	0.0 ± 1.5	14.3 ± 1.7	0.1 ± 1.9	14.5 ± 1.3	–0.8 ± 0.9	14.8 ± 1.4	–1.4 ± 1.7
18:1n-9	10.1 ± 1.5	0.2 ± 1.5	10.1 ± 1.7	0.1 ± 1.9	10.3 ± 1.1	–0.8 ± 0.7	10.4 ± 1.2	–1.4 ± 1.3[b]
PUFA	37.9 ± 1.9	–0.1 ± 2.0	37.7 ± 2.4	0.1 ± 2.5	38.3 ± 1.5	0.8 ± 1.5	38.2 ± 1.7	0.4 ± 1.8
18:2n-6	22.4 ± 2.4	0.2 ± 3.0	21.8 ± 2.4	–0.7 ± 3.1	23.6 ± 2.1	–0.3 ± 3.1	23.2 ± 2.6	–3.9 ± 2.5[b]
18:3n-6	0.1 ± 0.1	–0.0 ± 0.1	0.1 ± 0.0	–0.0 ± 0.0	0.1 ± 0.1	–0.1 ± 0.1	0.1 ± 0.1	–0.0 ± 0.1
18:3n-3	0.3 ± 0.1	0.0 ± 0.1	0.4 ± 0.1	0.0 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0.1 ± 0.1
20:3n-6	2.7 ± 0.5	0.1 ± 0.6	2.8 ± 0.9	–0.9 ± 0.5[c]	2.5 ± 0.5	–0.9 ± 0.5[c]	2.6 ± 0.7	–1.2 ± 0.6[c]
20:4n-6	6.6 ± 1.2	–0.2 ± 1.0	6.7 ± 1.1	–1.2 ± 0.5[b]	6.4 ± 1.0	–1.1 ± 0.8[a]	6.6 ± 1.3	–0.6 ± 0.8
20:5n-3	1.2 ± 0.4	–0.1 ± 0.4	1.4 ± 0.5	1.5 ± 1.4[b]	1.1 ± 0.3	0.4 ± 0.6[a]	1.1 ± 0.4	3.9 ± 1.1[c]
22:4n-6	0.2 ± 0.1	–0.0 ± 0.1	0.1 ± 0.1	–0.1 ± 0.0	0.1 ± 0.1	–0.1 ± 0.8	0.2 ± 0.1	–0.1 ± 0.1[a]
22:5n-3	0.6 ± 0.1	–0.0 ± 0.1	0.6 ± 0.1	0.1 ± 0.2	0.6 ± 0.1	–0.3 ± 0.1[c]	0.6 ± 0.1	0.3 ± 0.2[c]
22:6n-3	3.7 ± 0.5	–0.0 ± 0.7	3.8 ± 0.8	1.4 ± 1.2[b]	3.5 ± 0.9	3.0 ± 1.3[c]	3.4 ± 0.6	2.1 ± 0.8[c]
∑n-6	32.1 ± 2.1	–0.1 ± 2.6	31.5 ± 2.6	–2.8 ± 3.4[a]	32.7 ± 1.6	–2.3 ± 2.6[a]	32.6 ± 1.8	–5.7 ± 2.7[c]
∑n-3	5.9 ± 0.8	–0.1 ± 1.0	6.2 ± 1.3	3.0 ± 2.6[b]	5.6 ± 1.0	3.2 ± 1.8[c]	5.6 ± 0.8	6.2 ± 1.8[c]
Quantitative amounts (μg/mL)								
	Baseline	After 14 wk	Baseline	After 14 wk	Baseline	After 14 wk	Baseline	After 14 wk
20:5n-3	15.5 ± 6.0	13.2 ± 5.1	18.0 ± 7.2	34.4 ± 15.5	13.7 ± 3.0	19.1 ± 8.3[b]	16.5 ± 10.4	54.6 ± 18.8[c]
22:6n-3	51.2 ± 12.6	48.2 ± 11.4	50.0 ± 12.6	67.0 ± 8.4[b]	47.0 ± 12.6	85.1 ± 25.7[c]	49.5 ± 14.9	65.4 ± 20.2[b]

[a]At baseline and the changes after 14 wk, quantitative amounts of eicosapentaenoic and docosahexaenoic acids (μg/mL) at baseline and after 14 wk among the study groups. Values are means ± SD. Superscript letters a, b, c are significantly different from control group, $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively. See Table 1 for abbreviations.

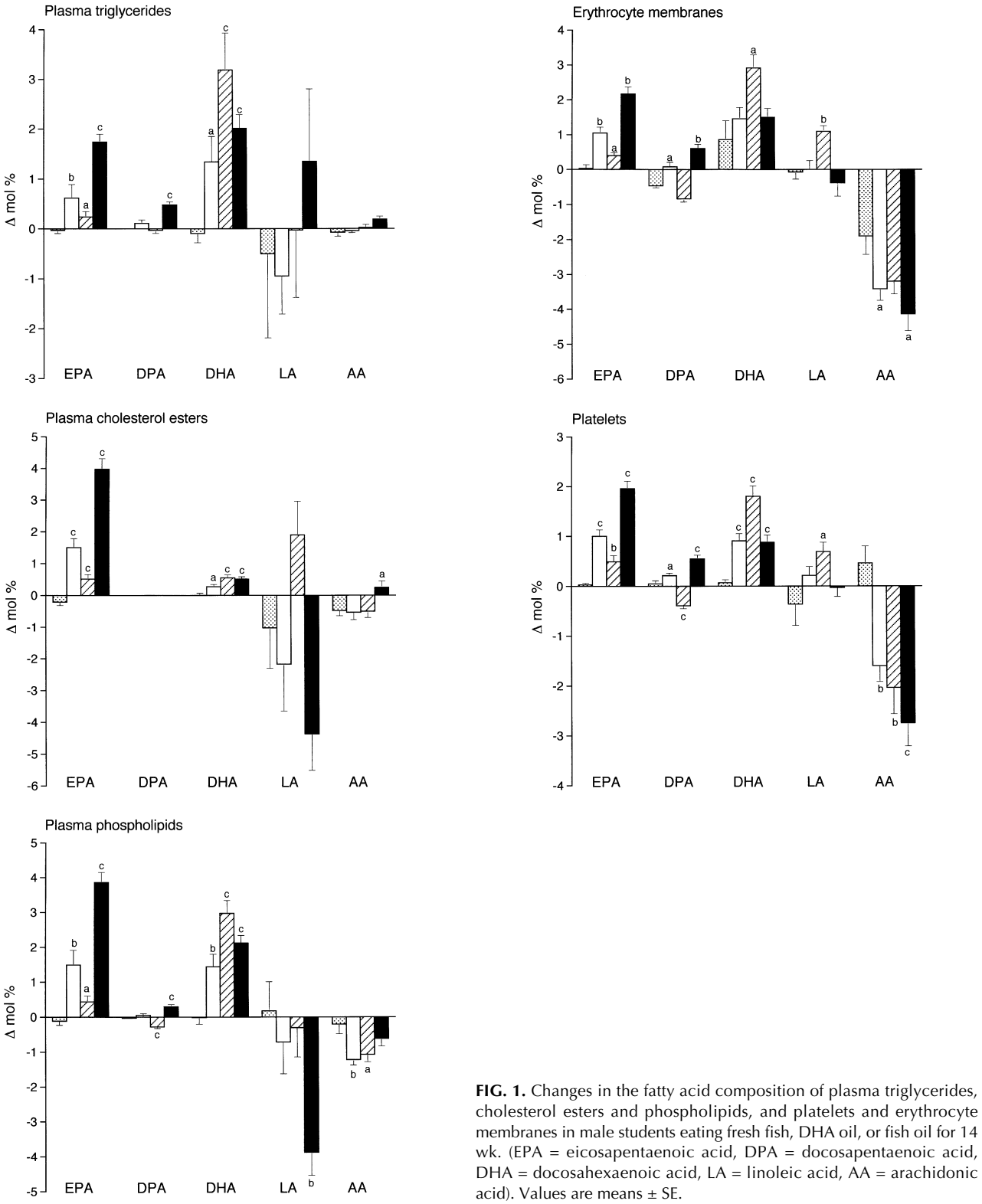

FIG. 1. Changes in the fatty acid composition of plasma triglycerides, cholesterol esters and phospholipids, and platelets and erythrocyte membranes in male students eating fresh fish, DHA oil, or fish oil for 14 wk. (EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid, DHA = docosahexaenoic acid, LA = linoleic acid, AA = arachidonic acid). Values are means ± SE.

lipids increased more than that of EPA, and this increase was greatest in PL and TG. Fish oil supplementation induced the most marked increase of EPA in PL and CE, whereas the increase of DHA was most evident in PL and TG. Figure 2 indicates the relationship between the intake of EPA and DHA and their levels in plasma PL. The same relationship applies to plasma TG, and platelets and erythrocytes membranes, as well (data not shown). Also the dose-response curves calculated on the basis of the results are shown in Figure 2. It should be noted that there are small amounts of EPA and

TABLE 5
Fatty Acid Composition (%mmol) of Platelets

	Control (n = 12)		Fish diet (n = 12)		DHA oil (n = 13)		Fish oil (n = 14)	
Fatty acid	Baseline	Change	Baseline	Change	Baseline	Change	Baseline	Change
SFA	44.1 ± 1.0	–0.2 ± 0.9	44.9 ± 1.7	–1.1 ± 1.2	44.0 ± 1.2	–0.8 ± 1.3	44.1 ± 1.2	–1.2 ± 1.1
MUFA	21.0 ± 0.9	–0.0 ± 0.8	20.6 ± 1.0	0.8 ± 0.8[a]	21.2 ± 1.5	0.5 ± 1.4	21.3 ± 0.8	1.2 ± 1.0[b]
18:1n-9	16.5 ± 0.8	0.0 ± 0.9	16.2 ± 0.8	0.5 ± 0.7	16.7 ± 1.2	0.5 ± 1.1	16.6 ± 0.6	1.1 ± 0.8[b]
PUFA	34.8 ± 0.7	0.3 ± 1.3	34.5 ± 1.9	0.2 ± 1.4	34.8 ± 1.8	0.3 ± 2.2	34.6 ± 1.0	–0.0 ± 1.0
18:2n-6	6.3 ± 1.4	–0.4 ± 1.5	5.9 ± 0.7	0.2 ± 0.6	6.3 ± 0.7	0.7 ± 0.6[a]	6.1 ± 0.6	–0.0 ± 0.7
20:3n-6	1.3 ± 0.2	0.1 ± 0.1	1.2 ± 0.3	–0.0 ± 0.2	1.3 ± 0.3	0.0 ± 0.2	1.3 ± 0.2	–0.0 ± 0.7[b]
20:4n-6	21.7 ± 1.3	0.5 ± 1.2	21.6 ± 1.5	–1.6 ± 1.1[b]	22.0 ± 1.4	–2.0 ± 1.9[b]	21.8 ± 0.9	–2.8 ± 1.7[c]
20:5n-3	0.7 ± 0.1	0.0 ± 0.1	0.7 ± 0.2	1.0 ± 0.5[c]	0.7 ± 0.2	0.5 ± 0.4[b]	0.7 ± 0.2	2.0 ± 0.6[c]
22:4n-6	1.1 ± 0.1	–0.0 ± 0.1	1.3 ± 0.2	–0.4 ± 0.1[c]	1.1 ± 0.2	–0.4 ± 0.2[c]	1.3 ± 0.3	–0.6 ± 0.3[c]
22:5n-3	1.1 ± 0.2	0.1 ± 0.2	1.2 ± 0.2	0.2 ± 0.2[a]	1.1 ± 0.2	–0.4 ± 0.2[c]	1.2 ± 0.2	0.6 ± 0.3[c]
22:6n-3	2.2 ± 0.3	0.1 ± 0.2	2.1 ± 0.5	0.9 ± 0.5[c]	1.9 ± 0.4	1.8 ± 0.8[c]	2.0 ± 0.2	0.9 ± 0.5[c]
∑n-6	30.4 ± 0.9	0.2 ± 1.2	30.0 ± 1.7	–1.8 ± 1.6[b]	30.6 ± 1.7	–1.6 ± 2.5[b]	30.4 ± 1.0	–3.4 ± 1.5[c]
∑n-3	4.0 ± 0.4	0.2 ± 0.4	4.0 ± 0.6	2.1 ± 1.0[c]	3.7 ± 0.5	1.9 ± 1.0[c]	3.8 ± 0.5	3.4 ± 0.9[c]

[a]At baseline and the changes after 14 wk among the study groups. Values are means ± SD. Superscript letters a, b, c are significantly different from control group, $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively. See Table 1 for abbreviations.

DHA derived from α-linolenic acid. Therefore, concentrations of EPA and DHA are clearly measurable even with zero dietary intake of these fatty acids. It seems that the response curves of EPA and DHA are steepest with low intakes of EPA and DHA, and the response curves follow logarithmic equations: the higher the intake the lower the proportional increase in concentration (Fig. 2).

Fatty acid composition of platelets. In platelets, the proportions of EPA and DHA were substantially increased mainly at the expense of arachidonic acid and docosatetraenoic acid in the fish diet, DHA oil, and fish oil groups (Table 5, Fig. 1). Also the proportion of docosapentaenoic acid increased in the fish diet and fish oil groups but was decreased by DHA oil supplementation. Both in the fish diet and fish oil groups the total proportion of MUFA and in the fish oil group the proportion of oleic acid increased significantly. In contrast to other groups, the proportion of linoleic acid increased in the DHA oil group.

Fatty acid composition of erythrocyte membranes. The proportions of EPA in erythrocyte membranes increased in the fish diet, DHA oil, and fish oil groups. The increase of DHA was statistically significant only in the DHA oil group (Table 6, Fig. 1). In the fish diet group, the proportions of arachidonic and docosatetraenoic acids were significantly decreased compared with those of the control group. The DHA oil group had a significantly increased proportion of linoleic acid and decreased proportion of docosatetraenoic acid. In the fish oil group, the proportion of docosapentaenoic acid increased and the proportions of dihomo-γ-linolenic, arachidonic, and docosatetraenoic acids decreased when compared with the control group.

The proportions of EPA and DHA in plasma PL correlated with those in platelets and erythrocyte membranes both at baseline (r = 0.50–0.72, $P < 0.001$ for EPA; and 0.52–0.65, $P = 0.001$ or less for DHA) and after 14 wk (r = 0.83–0.89; $P < 0.001$ and r = 0.40–0.78; $P = 0.015$ or less, respectively)

TABLE 6
Fatty Acid Composition (mol% of total) of Erythrocyte Membranes[a]

	Control (n = 6)		Fish diet (n = 11)		DHA oil (n = 10)		Fish oil (n = 12)	
Fatty acid	Baseline	Change	Baseline	Change	Baseline	Change	Baseline	Change
SFA	43.4 ± 0.5	0.7 ± 1.3	43.4 ± 0.9	1.2 ± 1.8	43.0 ± 0.8	0.3 ± 1.6	43.1 ± 0.7	1.1 ± 1.6
MUFA	22.5 ± 0.9	0.7 ± 0.5	23.1 ± 0.8	0.5 ± 2.0	22.9 ± 1.3	0.0 ± 1.3	23.0 ± 1.4	0.3 ± 2.7
18:1n-9	16.8 ± 1.1	–0.1 ± 1.0	17.5 ± 0.7	–0.2 ± 2.0	17.1 ± 1.2	–0.2 ± 1.2	17.1 ± 1.2	–0.4 ± 2.0
PUFA	34.0 ± 1.0	–1.4 ± 1.3	33.5 ± 0.8	–1.6 ± 1.5	34.1 ± 0.7	–0.3 ± 1.7	33.8 ± 1.1	–1.4 ± 1.7
18:2n-6	8.4 ± 0.7	–0.1 ± 0.5	8.6 ± 0.9	0.0 ± 0.8	9.0 ± 1.0	1.1 ± 0.5[b]	9.3 ± 1.0	–0.4 ± 1.3
20:3n-6	1.5 ± 0.2	0.1 ± 0.2	1.5 ± 0.3	–0.2 ± 0.2	1.5 ± 0.2	–0.2 ± 0.2	1.5 ± 0.3	–0.4 ± 0.2[b]
20:4n-6	16.5 ± 1.0	–1.9 ± 1.3	15.9 ± 1.2	–3.4 ± 1.1[a]	16.1 ± 1.4	–3.2 ± 1.2	15.2 ± 1.6	–4.1 ± 1.7[a]
20:5n-3	0.8 ± 0.2	0.0 ± 0.3	0.8 ± 0.3	1.0 ± 0.6[b]	0.7 ± 0.1	0.4 ± 0.3[a]	0.7 ± 0.2	2.2 ± 0.7[b]
22:4n-6	1.3 ± 0.2	–0.3 ± 0.2	1.5 ± 0.3	–0.6 ± 0.2[a]	1.4 ± 0.2	–0.6 ± 0.3[a]	1.6 ± 0.4	–0.8 ± 0.4[b]
22:5n-3	1.7 ± 0.2	–0.5 ± 0.2	1.7 ± 0.3	0.1 ± 0.4[a]	1.7 ± 0.3	–0.8 ± 0.3	1.8 ± 0.2	0.6 ± 0.4[b]
22:6n-3	3.3 ± 0.6	0.9 ± 1.3	3.0 ± 0.5	1.5 ± 1.0	3.2 ± 0.9	2.9 ± 1.2[a]	3.2 ± 0.6	1.5 ± 0.8
∑n-6	19.3 ± 1.0	2.1 ± 1.4	18.9 ± 1.4	–4.2 ± 1.1[a]	19.0 ± 1.6	–4.0 ± 1.2[a]	18.3 ± 1.8	–5.4 ± 2.0[b]
∑n-3	5.8 ± 0.7	0.4 ± 1.3	5.5 ± 0.9	2.6 ± 1.7[a]	5.6 ± 1.1	2.5 ± 1.4[a]	5.7 ± 0.9	4.3 ± 1.7[b]

[a]At baseline and the changes after 14 wk among the study groups. Values are means ± SD. Superscript letters a, b are significantly different from control group, $P < 0.05$, $P < 0.01$, respectively. See Table 1 for abbreviations.

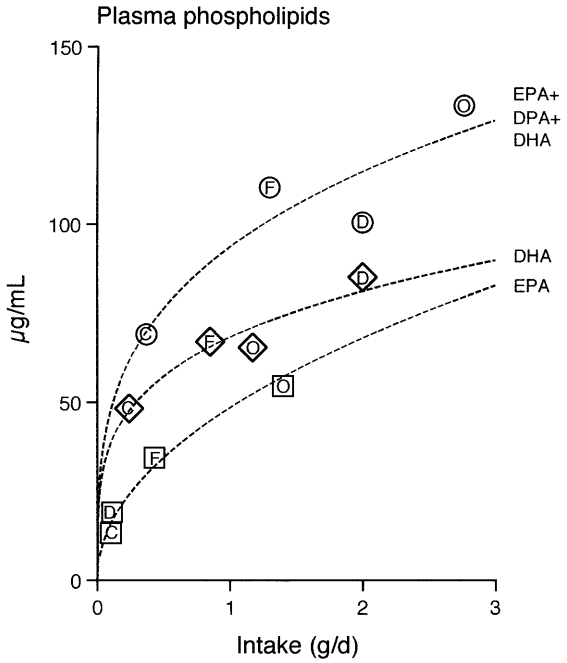

FIG. 2. Relationship between the total intakes (g/d) of EPA, DPA, and DHA (supplementation + dietary intake) and the concentrations in plasma phospholipids (μg/mL). C = control group, F = fish diet group, D = DHA oil group, O = fish oil group. See Figure 1 for other abbreviations.

among the study groups (study subjects pooled together). Correlations between the changes in the levels of EPA in plasma PL with those of EPA in erythrocyte membranes and platelets were $r = 0.91$, $P < 0.001$ and $r = 0.86$, $P < 0.001$, and the corresponding correlations between the levels of DHA were $r = 0.70$, $P < 0.001$ and $r = 0.77$, $P < 0.001$.

DISCUSSION

Fatty acid composition of the diet has a major impact on the fatty acid compositions in plasma lipids, platelets and erythrocyte membranes, but these fractions differ in their response to increased EPA and DHA intakes, as also shown in the present study when examining the effects of n-3 fatty acid supplementation, in the form of fresh fish, fish oil, and DHA oil, on the fatty acid composition of plasma lipid fractions, and platelets and erythrocyte membranes of young healthy male students. In the fish diet and fish oil groups, EPA was preferentially incorporated in plasma CE and PL, as shown in earlier studies, too (13,20,21). In contrast, the increase of DHA was greatest in plasma PL and TG in these groups, whereas only a small increase of DHA was seen in CE. This may be caused by the greater specificity of lecithin-cholesterol acyltransferase (LCAT) to EPA compared with DHA (21). Correlations between the fish intake of Finns (31 g/d in women and 37 g/d in men, respectively) and EPA content of serum CE ($r = 0.60$) and PL ($r = 0.52$) as well as in TG ($r = 0.36$), as reported by Nikkari and coworkers (22), support our results.

EPA and DHA levels in plasma PL have been used as an indicator of fish intake (23,24). Our study subjects were male students habitually consuming diets relatively low in fish products (26–35 g/d). The variability of EPA in plasma PL has been explained by habitual fish consumption, but fish consumption explains a lower proportion of variability in DHA in plasma lipids (25,26). Svensson and coworkers (27) found fourfold higher EPA and twofold higher DHA content of serum phosphatidylcholine in men who consumed fatty fish at an average of 1046 g/wk compared with men who did not consume fish at all. The proportions of both EPA and DHA correlated significantly with fish intake. In the present study, the increase of EPA in plasma PL was about 1.7 times greater compared with that of DHA in the fish diet and fish oil groups. The reason for this could be a more efficient liberation of DHA from chylomicrons (28), leaving more EPA in chylomicron remnants and thereby making more EPA available for PL synthesis in liver.

In addition, the data reported here suggest that the proportions of EPA in platelets reflect well the habitual fish intake over a long term. EPA in platelets (wt%), but not DHA, has been reported to correlate with habitual fish consumption (26). However, in our study the effect of increased DHA intake was seen in platelets, and the change correlated strongly with that of plasma PL. Thus, it is possible that DHA also reflects fish intake, but its less steep dose-response curve compared to EPA makes it more difficult to find strong correlations especially when intakes are estimated from questionnaires. In the fish oil group, the changes in the proportions of EPA and DHA in platelets and erythrocytes were similar as observed after 6-wk cod liver oil supplementation (29), but the change in the proportion of DHA in erythrocytes was greater in the present study. This stresses the importance of the duration of supplementation when erythrocytes are examined.

Our results indicate that the response curves are steepest with low intakes of EPA and DHA (Fig. 2). This view is supported by the results of Bønaa and coworkers (25) comparing plasma PL fatty acids and habitual fish intake, and also by the dose-response study with four different fish intake levels (30). In a dose-response study, the increase of EPA + DHA intake from 3 to 6 g/d caused a very modest increase in their amount in plasma PL compared with the effect of increasing the intake from 1.5 to 3 g/d (31), showing only a weak effect of very high doses. Thus, it seems that the dose-response curves for both EPA and DHA follow logarithmic equations. DHA intake alone does not increase the total amount of n-3 fatty acids as much as the combination of EPA, docosapentaenoic acid, and DHA. In addition, the results of the fish oil group suggest that the high amount of EPA in this product (EPA to DHA ratio 1.4) decreases the effect of DHA when compared with the fish diet group (EPA to DHA ratio 0.6). These results indicate that the relationship between the intake of EPA and DHA and their amounts in plasma and membranes is dependent on the ratio of these fatty acids in the diet, and a reliable equation cannot be calculated on the basis of these results. To apply plasma or membrane EPA and DHA levels for the estimation of fish intake, dose-responses should be determined

using different fish intake levels, and dose-responses for other n-3 fatty acid products should be determined separately.

Interestingly, DHA oil supplementation caused an increase in the proportion of EPA both in plasma lipid fractions and in platelets and erythrocyte membranes, although DHA oil did not contain EPA at all, and the intake of EPA from habitual diet was on average only 0.09 ± 0.04 g/d during the study. This gives evidence of retroconversion of dietary DHA to EPA. Retroconversion of DHA to EPA takes place in peroxisomes by Δ4 enoyl reductase and Δ3, Δ2 enoyl CoA isomerase enzymes (32,33). Retroconversion of DHA to EPA has also been documented in some human (13) and animal studies (34–37), and also *in vitro* (33,37,38). In these studies the increase of docosapentaenoic acid has also been noticed. Docosapentaenoic acid could be a result of a saturation of the DHA or a chain elongation of EPA or both (32,33). However, in the present study the amount of docosapentaenoic acid was unchanged in plasma TG; it decreased in plasma PL, and in platelets and erythrocyte membranes it decreased as much as EPA increased. The decrease of docosapentaenoic acid has also been reported in a short-term study by von Schacky *et al.* (8) in plasma and platelet PL in humans after high DHA ingestion. The decrease of docosapentaenoic acid in the DHA oil group may indicate that the increase of EPA after DHA supplementation is not caused solely by retroconversion but could be a consequence of decreased elongation of EPA as well.

In our fish diet group, a reduced level of arachidonic acid in plasma PL, platelets, and erythrocyte membranes was noticed compared with the control group. Earlier studies have demonstrated that fish consumption decreases the concentration of n-6 PUFA in plasma (26,39), and a negative correlation between fish intake and the proportion of arachidonic acid in serum lipids has been observed (40,41). Eskimos who have a diet high in n-3 fatty acids have low levels of arachidonic acid in serum lipids (7,42). Siess *et al.* (5) reported a considerably reduced arachidonic acid proportion in platelet PL after consumption of a mackerel diet with high doses of EPA (7–11 g/day for 8 d), but also an unchanged proportion of arachidonic acid in plasma (10,32) and platelet PL (10) has been reported. It has been speculated that an unaltered arachidonic acid proportion could be a consequence of transforming linoleic acid to arachidonic acid (40), and the reduced level of arachidonic acid in platelets, but not in serum PL or total serum lipids, could be due to a direct competition between arachidonic acid and n-3 fatty acids for acylation into PL (33).

An interesting finding in our study after DHA supplementation was that the proportion of linoleic acid increased in platelets and erythrocyte membranes and remained unchanged in plasma fractions. DHA has been noticed to decrease Δ6 desaturase activity in rats (43) and to inhibit Δ6 desaturase *in vitro* (44). Our results support these findings and suggest that the transformation of linoleic acid to its metabolites is decreased by DHA. In addition to this step, Δ6 desaturation is also involved in the pathway of DHA from docosapentaenoic acid *via* 24:5n-3 and 24:6n-3. Thus it is possible that the inhibition of this desaturase contributes to the observed increase of the proportion of EPA and linoleic acid and decrease of that of docosapentaenoic acid after DHA supplementation.

In conclusion, fish diet and fish oil supplementation increased the proportions of n-3 fatty acids in plasma lipids, platelets, and erythrocyte membranes. The most striking differences in the distribution of EPA and DHA were seen in plasma CE with preferential incorporation of EPA and in plasma TG with preferential incorporation of DHA. DHA supplementation increased also the proportion of EPA, but in contrast to fish diet and fish oil supplementation, the proportion of docosapentaenoic acid remained unchanged or even decreased in fractions analyzed. Another special aspect of DHA supplementation was the increased proportion of linoleic acid in platelets and erythrocytes. These results indicate that there are marked differences in the effects of EPA and DHA on the composition of plasma and membrane lipids.

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Heneicosapentaenoate (21:5n-3): Its Incorporation into Lipids and Its Effects on Arachidonic Acid and Eicosanoid Synthesis

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ABSTRACT: 6,9,12,15,18-Heneicosapentaenoic acid (21:5n-3) (HPA), present in small amounts in fish oils, has been prepared by chemical elongation of eicosapentaenoic acid (EPA) and its biological properties compared with EPA and docosahexaenoic acid (DHA). All the double bonds of HPA are displaced one carbon away from the carboxyl group when compared to EPA. HPA is incorporated into phospholipids and into triacylglycerol in cell culture to a similar extent as EPA and DHA. HPA is a stronger inhibitor of the conversion of α-linoleic acid and dihomo-γ-linolenic acid to arachidonic acid (AA) in hepatoma cells than are EPA, DHA, and AA. HPA is a poor substrate for prostaglandin H synthase and for 5-lipoxygenase, but it inactivates prostaglandin H synthase as rapidly as do AA, EPA, and DHA. HPA inhibits thromboxane synthesis in isolated platelets as efficiently as EPA. EPA, HPA, and DHA are all weak inducers of acyl-CoA oxidase in hepatoma cells. Therefore, since fish oils contain only small amounts of HPA, it is unlikely that this fatty acid is of particular significance for the biological effects of these oils, possibly with the exception that it is a strong inhibitor of AA synthesis.
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Fish oils are known to contain very long n-3 polyunsaturated fatty acids, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Small amounts of a third, 21-carbon chain length, n-3 polyunsaturated fatty acid, heneicosapentaenoic acid (HPA), has also been identified (1). The origin of this fatty acid is uncertain, but α-oxidation and decarboxylation of 7,10,13,16,19-docosapentaenoic acid (22:5n-3) have been suggested (2). Chemically it is an EPA elongated with one carbon in the carboxyl end. This elongation places the first double bond in the Δ6 position, which is different from that of both EPA (Δ5) and DHA (Δ4). This acid therefore is interesting both because it is found in nature and because it can be used to study the significance of the position of the double bonds in n-3 fatty acids.

The contribution of HPA to the biological effects of fish oils is unknown. Only in one study has synthetic HPA been shown to be a poor substrate of 5-lipoxygenase (3). Considering the many biological effects of fish oil and of its EPA and DHA, we found it of general interest to compare the biological properties of HPA with those of the more abundant EPA and DHA. In the present study, we have tested this fatty acid for its incorporation into phospholipids and triglycerides; we have also compared the effects of EPA, HPA, and DHA as substrates and inhibitors of PGH-synthase and 5-lipoxygenase, which initiate the synthesis of prostaglandins, thromboxane, and leukotrienes. We also have studied the effects of HPA on the conversion of linoleic acid (18:2n-6) to arachidonic acid (AA) in hepatoma cells. The first, rate-limiting enzyme in this conversion is Δ6 desaturase. In all the experiments, EPA and DHA have been included for comparison.

MATERIALS

[1-^{14}C] AA (50–60 mCi/mmol) and [1-^{14}C] linoleic acid (50 mCi/mmol) were obtained from Amersham Laboratories (Buckinghamshire, England). [1-^{14}C]Dihomo-γ-linolenic acid (40–60 mCi/mmol) was from New England Nuclear (Boston, MA). AA and other polyunsaturated fatty acids as well as reduced glutathione, hydroquinone, prostaglandin (PG)-/leukotriene standards, phenylmethylsulfonyl fluoride, trypsin inhibitor Type II-S: Soybean, ATP, and Tris were all purchased from Sigma (St. Louis, MO). EPA and docosapentaenoic acid were gifts from Norsk Hydro (Porsgrunn, Norway). 2,7-Dichlorofluorescein diacetate was from Eastman Kodak Company (Rochester, NY). Picofluor^{TM} 40 was obtained from Packard Instrument Company, Inc. (Groningen, The Netherlands). Dulbecco's minimum essential medium w 74500 mg/L, McCoys 5A medium, Ham's F10 medium, glucose, sodium pyruvate, ultroser and gentamicin, anti- PPLO (a concentrated preparation of the antibiotic Tylocine, used against pleuropneumonia-like organisms), fungizone, penicillin, and streptomycin were from Gibco (Grand Island, NY). Plastic culture dishes and bottles were obtained from Costar (Cambridge, United Kingdom).

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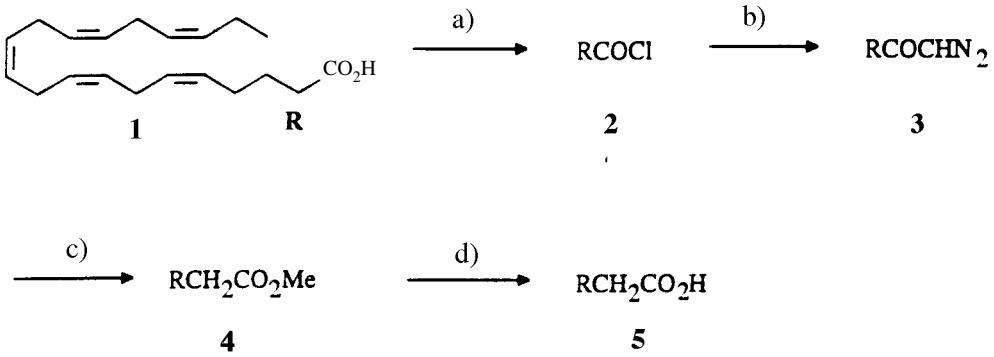
Abbreviations: 20:4n-6, AA (arachidonic acid); ANOVA, analysis of variance; BHT, butylated hydroxy toluene; 22:6n-3, DHA (docosahexaenoic acid); 5,12-diHETE, 5,12-dihydroxyeicosatetraenoic acid; 20:5n-3, EPA (eicosapentaenoic acid); 5-HETE, 5-hydroxyeicosatetraenoic acid; 21:5n-3, HPA (heneicosa-pentaenoic acid); 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; RP-HPLC, reversed phase high performance liquid chromatography; LT, leukotriene; PBS, phosphate-buffered saline; PG, prostaglandin.



Reagents and conditions: a) $(COCl)_2$/PhH 1.5 h rt., b) CH_2N_2/ether 20 min. 0°C, c) BzAg (cat.), Et_3N/CH_3OH 1 h rt., d) guanidine/LiOH.

Synthesis of all-cis-*6,9,12,15,18-heneicosapentaenoic acid* (Scheme 1).

General. All reactions, workup included, were carried out in the dark under nitrogen. The reactions were monitored by thin-layer chromatography; the following systems were used as eluent: ethyl acetate/hexane 1:4 (**I**), 1:6 (**II**), 4:96 (**III**), 1:1 containing 0.5% acetic acid (**IV**), and hexane saturated with acetonitrile (**V**). All standard reagents used were either from Fluka (Buchs, Switzerland) or Janssen (Acros Chimica, Grel, Belgium), the solvents were from Fluka or Merck (Darmstadt, Germany). Generally, solvents were removed at 10 mm Hg and finally under high vacuum for ≥2 h. Alcohol-free diazomethane was generated in a Mini Diazald Apparatus from Aldrich (Milwaukee, WI).

all-cis-*5,8,11,14,17-Eicosapentaenoyl chloride* (**2**). Oxalyl chloride (2.0 mL, 27 mmol) was added to a stirred benzene solution of *all-cis*-5,8,11,14,17-EPA (2.75 g, 9.1 mmol) kept at 20°C. After 1 h 30 min, volatile material was removed at reduced pressure. The evaporation was repeated twice with more anhydrous benzene (2 × 10 mL). The acid chloride **2**, obtained as a straw-colored oil, was dissolved in dry ether (16 mL) and used without purification in the next step.

all-cis-*Diazo-6,9,12,15,18-heneicosapentaen-2-one* (**3**)(Ref. 4). A solution of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (7.7 g, 35 mmol) in ether (70 mL) was added to a mixture of potassium hydroxide (4.2 g), water (7 mL), 2-(2-ethoxyethoxy) ethanol (22 mL) and ether (14 mL) kept at 65°C within 35 min. Additional ether (16 mL) was used as rinse. The ether was condensed and collected at −78°C, and the resulting etheral solution of diazomethane was quickly filtered through a wad of cotton.

The etheral solution of the acid chloride **2**, cooled to −5–0°C, was added within 1 min to a vigorously stirred solution of diazomethane (approximately 25 mmol) in ether (approximately 100 mL) kept on an ice bath. Dry ether (2 × 4 mL) was used as rinse. A brisk evolution of gas lasted for 10 min. After 20 min all volatile material was removed at 200–10 mm Hg and ≤0°C to give 2.88 g of crude product. Excess diazomethane was conveniently destroyed by means of a gas washing flask (with fritted disc) containing a small amount of acetic acid connected between the reaction flask and the pump.

Purification by flash chromatography (pyridine-treated silica 180 mL, ethyl acetate/hexane 1:9 800 mL, 1:6 200 mL; approximate elution volume 360–830 mL) afforded 2.60 g (88% from **1**) bright orange-colored diazoketone **3**. Infrared (IR); 3013, 2104, 1646 cm^{-1}.

Methyl all-cis-*6,9,12,15,18-heneicosapentaenoate* (**4**)(Refs. 5,6). A solution of silver benzoate (120 mg, 0.52 mmol) in triethylamine was added to a stirred solution of diazoketone **3** (2.0 g, 6.13 mmol) in dry methanol (10 mL) kept at room temperature within 1 h. The reaction was quenched by the addition of hydrochloric acid (1 N, 0.6 mL) and the solvents removed at room temperature under reduced pressure. The residue was partitioned between hexane (30 mL) and hydrochloric acid (1 N, 6 mL). The organic phase was washed with aqueous sodium bicarbonate (10%, 6 mL) and worked up to give 1.96 g crude ester. Purification by flash chromatography (silica 200 mL; ethyl acetate in hexane 2% 400 mL, 3% 600 mL, 4% 400 mL; approximate elution volume 320–770 mL) afforded 1.81 g (89%) of methyl ester **4** as a colorless oil. IR; 3013, 1742 cm^{-1}.

all-cis-*6,9,12,15,18-Heneicosapentaenoic acid* (**5**). The methyl ester **4** (1.70 g, 5.14 mmol) was saponified with guanidine hydrochloride (1.72 g, 18 mmol) and lithium hydroxide monohydrate (504 mg, 12 mmol) in 80% ethanol (20 mL) at room temperature. After 6 h the reaction mixture was washed twice with hexane (2 × 10 mL). The hexane fractions were washed successively with brine (5 mL) that was added with stirring to the reaction mixture, followed by hydrochloric acid (1 N, 30 mL). The free fatty acid was extracted with hexane (40 + 2 × 20 mL) and evaporated to give 1.46 g (90%) of the product **5** as a slightly flaxen oil. IR; 3013, 1709 cm^{-1}. ^1H nuclear magnetic resonance (NMR) (300 MHz, $CDCl_3$); δ; 0.98 (3H, *t*, CH_3), 1.45 (2H, *m*, CH_2), 1.65 (2H, *m*, CH_2), 2.1 (4H, *m*, CH_2-C=), 2.37 (2H, *t*, CH_2-CO), 2.82 (8H, *m*, =C-CH_2-C=), 5.35 (10H, *m*, CH=CH). ^{13}C NMR (75 MHz, $CDCl_3$); δ; 179.92 (CO), 131.89, 129.43, 128.42, 128.18, 128.09, 127.98, 127.91, 127.08, 127.74, 126.88 (C=C), 33.83, 28.85, 26.71, 25.51, 25.41, 24.14, 20.43 (CH_2), 14.14(CH_3).

The conversion of EPA to HPA by this procedure was performed with an all-over yield of approximately 70%.

Cell culture. 7800 C1 Morris hepatoma cells were cultivated mainly as described by Richardson *et al.* (7). They were grown

to confluence in Ham's F 10 medium supplemented with 10% horse serum and 3% calf serum. The medium was supplemented with penicillin (50 U/mL), streptomycin (50 µg/mL), fungizone (2.5 µg/mL), and anti-PPLO (60 µg/mL), and changed every 48 h. The cells were harvested in 1–2 mL phosphate-buffered saline (PBS) by using a rubber policeman.

Rat hepatocytes were prepared from male Wistar rats (350 g), obtained from Møllegaard Breeding Center (Ejby, Denmark). After barbiturate anesthesia, liver parenchymal cells were isolated as described by Berry and Friend (8) with modifications according to Seglen (9). The hepatocytes were plated in 6 × 15 mm culture dishes overnight in DME medium containing HEPES (20 mM), Ultroser G (2%), and gentamicin (50 µg/mL) at a cell density of approximately 2 million cells/dish (1–1.5 mg cell protein) in a volume of 6 mL. The cells were incubated at 37°C in 5% CO_2 and 95% air for 18–20 h before use.

Mouse peritoneal macrophages were isolated mainly as described by Stuart (10) and cultivated in McCoys 5A medium, containing 20 µM fatty acid and 1% fetal calf serum, for 3–18 h.

ASSAY METHODS

Isolation of phospholipids and triacylglycerol. Lipids were extracted from harvested cells twice with butanol (1 mL butanol per 2 mL water phase)(11). L-α-Phosphatidylcholine-di-heptadecanoyl (44 µg) was added as internal standard. The butanol was evaporated under a stream of nitrogen, and the residues were dissolved in chloroform containing 1 mg of butylated hydroxytoluene as antioxidant. Each sample was then subjected to anion-exchange chromatography on bonded-phase columns (12), to yield triacylglycerol and phospholipid fractions.

Gas chromatography of fatty acids. Fatty acids were transmethylated (13) and separated by capillary gas chromatography on a 50 m × 0.22 mm BP1-polyimide coated fused-silica capillary column, film thickness 0.25 µm (SGE M9 column; S.G.E. International, Ringwood, Victoria, Australia) using a Shimadzu GC-14A model gas chromatograph (Tokyo, Japan), fitted with a 3390 A Hewlett-Packard integrator (Palo Alto, CA). Helium at 2.5 kg/cm^2 was used as carrier gas, and the split/splitless injector was used in the split mode with a ratio of 60:1. After sample injection, the temperature was initially held for 5 min at 140°C, then programmed to increase with 4°/min to 250°C and held there for 30–45 min. Amounts of fatty acids were calculated by using the internal standard method and previously determined response factors.

Conversion of 1-^{14}C linoleic- and 1-^{14}C dihomo-γ-linolenic acid. Confluent 7800 C_1 Morris hepatoma cells in 6-cm (diameter) dishes were incubated with [1-^{14}C]linoleic acid (50 µM) and inhibitory fatty acids (50 µM) for 18 h. Cells were then washed with PBS and harvested in 2 mL PBS. The lipids were extracted and methylated as described in the paragraph on gas chromatography and run on high-performance liquid chromatography (HPLC) (model SP 8800 with a Spectra 100 variable wavelength detector and a Raytest Ramona 5 LS liquid-scintillation counter with a 1.0 mL flow cell on line; Spectra-physics). The dry residues were dissolved in 0.2 mL 80% acetonitrile and injected on a 4.6 × 250 mm column containing 5 µm Supelco RP-18 (Bellefonte, PA). Linoleic acid (and dihomo-γ-linolenic acid) was separated from AA by running 80% acetonitrile for 30 min, with a flow rate of 2 mL/minute.

The inhibition of the Δ5 desaturation step was assayed by incubation of hepatoma cells with [1-^{14}C]dihomo-γ-linolenic acid instead of linoleic acid as above. The incubation time was 4 h.

The Δ6 desaturation step was assayed in isolated rat liver microsomes as described by Christiansen *et al.* (14). Microsomal protein (1.0 mg) was incubated for 15 min at 37°C with 25 µM [1-^{14}C]linoleic acid, with or without inhibitory fatty acid (25 µM). The incubation was stopped by addition of 1.0 mL of 15% KOH in methanol followed by heating at 65°C for 45 min. The samples were acidified with 6.0 N HCl and extracted with 3.0 mL of hexane. The hexane was evaporated under nitrogen and the residues were dissolved in 1.0 mL benzene. To methylate the fatty acids, 1.0 mL of methanol-HCl was added to extracted lipids dissolved in 1 mL of benzene and left for 30 min in a water bath at 37°C using capped tubes. The methylated fatty acids were extracted with 2.0 mL of hexane and assayed on HPLC as above.

PGH-synthase. Sheep seminal vesicles were obtained from a local slaughterhouse and stored at −70°C. Microsomal prostaglandin synthase was prepared as described by Egan *et al.* (15). The crude enzyme was stored in small batches at −70°C in 0.1 M potassium phosphate buffer (pH 7.5) (40 mg protein/mL). The enzyme was diluted 1:1 with 0.4% Triton X-100 before use.

Assay by oxygen consumption was recorded with a Clark oxygen electrode (MSE Scientific Instrument, Crawley, Sussex, England) in 0.1 M Tris buffer (2 mL, pH 8) with 0.33 mM glutathione and 0.5 mM phenol at 30°C. Reaction was started by addition of either fatty acid or enzyme (63 µg protein). Oxygen concentration in the buffer in equilibrium with air was assumed to be 221 nmoles O_2/mL at 30°C (16). Based on the stoichiometric reaction of 2 moles of O_2 per mole of fatty acid, the amount of fatty acid consumed during the reaction was calculated.

Assay by cosubstrate oxidation was based on the oxidation of leuco-dichlorofluorescein as reducing cosubstrate in the conversion of AA to PGH_2 as previously described (17).

Thromboxane B_2 production. Human blood platelets were isolated from volunteers essentially as described by Lagarde *et al.* (18). The volunteers had not taken any drugs for at least 10 d. AA (final concentration 10 µM) was used as an inducer of thromboxane production (19), and the reaction was stopped by centrifugation. Thromboxane B_2 produced was measured with an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) (20).

5-Lipoxygenase. RBL-1 cells were grown in suspension culture essentially as described by Kulczycki *et al.* (21). Cells were harvested and 5-lipoxygenase isolated as described by Haurand and Flohe (22). 5-Lipoxygenase activity was measured as oxygen consumption, monitored by a Clark oxygen electrode as described for cyclooxygenase.

The oxygraph chamber (2 mL) contained 8 mM ATP, 3 mM $CaCl_2$, 3 mM reduced glutathione, 50 mM Tris (pH 7.5) and enzyme (5×10^7 cell equivalents). Fatty acid was added as substrate (100 µM final concentration), and oxygen consump-

tion was followed for 2–3 min. The initial reaction rate was estimated from the initial slope of the oxygraph curve.

After incubations with [1-^{14}C] AA, analysis by reversed-phase HPLC (see later) showed formation of 5-hydroxyeicosatetraenoic acid (5-HETE) and 5,12-dihydroxyeicosatetraenoic acid (5,12-diHETE). No PGH-synthase products were detected.

5-Lipoxygenase products. The 5-lipoxygenase (2.5×10^6 cell equivalents) was incubated as above (22) with 20 µM [1-^{14}C]AA at 37°C and with or without 50 µM DHA, EPA, or HPA to study their inhibitory effects on conversion of radioactive AA. The reaction was stopped after 10 s by adding 500 µL acetone + two drops of HCOOH. The reaction products were extracted with $CHCl_3$. The chloroform was evaporated under a stream of N_2, and the dry residues were stored at –20°C until analyzed by reversed-phase HPLC.

The dry residues were dissolved in 0.1 mL of methanol/water (3:1, vol/vol)(see below), and injected on a 4.6×250 mm column containing 5 µm Supelco RP-18. (HPLC system, see earlier mention). Separation was achieved by running a 30-min linear gradient from 75 to 100% methanol (25–0% water containing 0.1% acetic acid, adjusted to pH 5.4 with ammonia) at a flow rate of 1 mL/min. EDTA was added to the eluent at 1 mM concentration. Unlabeled standards (10 µg), added to the samples, were detected at 236 nm (23). The retention time of 5,12-diHETE was 6–7 min, 5-HETE/5-hydroperoxyeicosatetraenoic acid (5-HPETE), 14–15 min, and AA, 24–25 min. To separate PGH-synthase products, a gradient from 60 to 100% methanol was used. Ultraviolet absorption at 206 nm was recorded. The ratio of eluent to scintillation fluid was set at 1:7 (vol/vol).

Other assays. Palmitoyl-CoA oxidase activity was measured essentially as described by Small *et al.* (24) with minor modifications (25). Protein was measured by the method of Lowry (26). Cellular DNA was measured according to Labarca and Paigen (27).

Statistics. Ordinary one-way analysis of variance (ANOVA) was performed and separate *P* values were calculated by Student's *t*-test.

RESULTS

Incorporation into phospholipids and triglycerides. Long-chain n-3 fatty acids normally are found in the phospholipids. We have studied the incorporation of HPA, compared to EPA and DHA, into hepatocyte phospholipids and triglycerides. Neither HPA, EPA, nor DHA had any observable toxic effects on the hepatocytes under the conditions used (fatty acid/albumin ratio, approximately 2.5:1).

Table 1 shows that the amounts of incorporated EPA, DHA, and HPA are similar. Under the conditions used, the incorporation is higher in triglycerides than in the phospholipids. A decrease of the oleic acid (18:1n-9) and linoleic acid (18:2n-6) content is observed in the phospholipids, whereas only oleic acid is decreased in the triglycerides after the treatment. Analyzing the changes in oleic acid content in phospholipids and triglycerides with the ANOVA test gave a *P* value of 0.0002 for oleic acid in phospholipids and 0.0001 in triglycerides which showed that the variation among column means was significantly greater than expected by chance. Also the variation in 18:2n-6 in phospholipids after treatment with fatty acids was significant with a *P* value of 0.0001. The other fatty acids are not significantly changed ($P > 0.05$). Also in 7800 C1 hepatoma cells (not shown), HPA was incorporated to a similar extent as DHA, while EPA was less incorporated. In hepatoma cells, all three fatty acids were incorporated mainly at the expense of AA (20:4n-6).

Other experiments showed that HPA, EPA, and DHA also are incorporated to a similar extent into mouse peritoneal macrophage-phospholipids (not shown).

Effect on linoleic acid conversion to AA. Feeding fish oils decreases the Δ6 desaturation of linoleic acid and its conversion to AA (14). Table 2 shows that HPA decreases the conversion of both linoleic acid and dihomo-γ-linolenic acid to AA even more than EPA and DHA in hepatoma cells. ANOVA test gave very

TABLE 1
Incorporation of EPA, HPA, and DHA into Hepatocyte-Phospholipids and -Triglycerides[a]

Fatty acid added	Percentage of fatty acids in A: phospholipids/B: triglycerides					
	18:1	18:2	20:4	20:5	21:5	22:6
A						
0	8.1 ± 1.5	17.3 ± 2.8	25.7 ± 4.0	n.d.	n.d.	3.9 ± 1.2
20:5	5.5 ± 1.1**	11.8 ± 0.9***	24.3 ± 1.7	10.3 ± 2.6	n.d.	3.7 ± 1.1
21:5	5.9 ± 0.9*	13.4 ± 0.9***	27.1 ± 2.9	n.d.	6.2 ± 3.4	3.6 ± 1.3
22:6	5.5 ± 1.2*	11.9 ± 0.9***	29.9 ± 4.4	1.4 ± 1.8	n.d.	9.6 ± 1.1***
B						
0	25.9 ± 2.7	18.8 ± 11.1	3.7 ± 2.6	n.d.	n.d.	1.4 ± 0.8
20:5	18.1 ± 2.2***	22.9 ± 7.4	5.7 ± 1.9	14.3 ± 9.3	n.d.	2.2 ± 0.9
21:5	17.4 ± 2.5***	20.7 ± 9.0	3.8 ± 1.8	n.d.	20.4 ± 8.4	1.6 ± 0.9
22:6	17.4 ± 1.9***	21.3 ± 8.5	5.1 ± 1.8	2.2 ± 0.9	n.d.	18.1 ± 5.9***

[a]Plated rat hepatocytes (1–1.5 mg cell protein per dish) were incubated for 24 h with or without 80 µM of eicosapentaenoic acid (EPA), heneicosapentaenoic acid (HPA), or docosahexaenoic acid (DHA) and 32 µM bovine serum albumin (final concentrations). The medium was changed after 8 h. The cells were washed with phosphate-buffered saline (PBS) and harvested in PBS; A: percentage of total fatty acids in phospholipids; B: percentage of total fatty acids in triglycerides. Results are shown as mean of three experiments, each with 2–3 replicates, ± SD; *P* values are related to control, *$P < 0.01$ **$P < 0.005$ ***$P < 0.001$. n.d., not detectable.

TABLE 2
Inhibition of Δ6 and Δ5 Desaturase Activity by 20:4n-6, 20:5n-3, 21:5n-3, and 22:6n-3[a]

Inhibitor	Microsomes 18:2 ⇒ 18:3[b]	Hepatoma cells 20:3 ⇒ 20:4	18:2 ⇒ 20:4[c]
Control	8.2 ± 0.6	10.2 ± 0.6	13.3 ± 0.5
20:4	7.6 ± 0.8[a]	4.9 ± 0.1**[bb]	6.1 ± 0.5**[bb]
20:5	5.6 ± 0.1*	4.9 ± 0.4**[b]	3.1 ± 0.4**[b]
21:5	6.7 ± 1.0	3.3 ± 0.3**	1.4 ± 0.4**
22:6	6.6 ± 0.4[a]	6.7 ± 0.7*[bb]	2.8 ± 0.1**[b]

[a]The enzyme activity of Δ6-desaturase (18:2 ⇒ 18:3) was measured in isolated rat microsomes, as γ-linolenic acid produced [percentage of added [1-^{14}C]-linoleic acid (18:2n-6)]. The activity of Δ5 desaturase (20:3 ⇒ 20:4) was measured in hepatoma cells as arachidonic acid (20:4n-6) produced (percentage of incorporated [1-^{14}C]-dihomo-γ-linolenic acid (20:3n-6)). The conversion of 18:2n-6 to 20:4n-6 was measured in hepatoma cells as arachidonic acid produced—percentage of incorporated [1-^{14}C]-linoleic acid (see Assay Methods section). The uptake of linoleic acid or dihomo-γ-linolenic acid into the cell lipids was not changed by the added unlabeled fatty acids. Each value is the mean of three replicates ± SD; *$P < 0.005$, **$P < 0.001$ vs. control; a: $P < 0.01$ vs. 20:5. b: $P < 0.005$, bb: $P < 0.001$ vs. 21:5.
[b]Percentage of 18:2 added to the reaction mixture.
[c]Percentage of substrate taken up by the cells.

significant *P* values for all three columns. In three different experiments on the conversion of linoleic acid to AA, we observed increased accumulation of dihomo-γ-linolenic acid in the hepatoma cells incubated with inhibiting n-3 fatty acids. This accumulation was most pronounced with HPA, but accurate quantification was not possible. The conversion of dihomo-γ-linolenic acid involves only Δ5 desaturase. The Δ6 desaturase cannot be assayed separately in intact cells because of subsequent elongation and Δ5 desaturation. The effect on this step therefore was measured in isolated microsomes (Table 2, column 1). In this assay, HPA showed only insignificant inhibition. Only EPA gave a moderate but significant inhibition compared to control.

PGH-synthase. PGH-synthase has two enzyme activities: cyclooxygenase which consumes two moles of O_2 per mole AA, and a peroxidase which reduces a perhydroxy group into a hydroxyl group with concomitant oxidation of a reducing cosubstrate. We have measured these two enzyme activities by two separate methods. The activity of the cyclooxygenase part was measured as O_2 consumption. Table 3 shows that EPA is only half as good a substrate as AA. DHA and HPA are poor substrates. We obtained an almost identical relative substrate specificity by using the spectrophotometric assay of cosubstrate oxidation (not shown).

Figure 1A shows that EPA, HPA, and DHA are weak competing substrates/inhibitors of cyclooxygenase, when added concomitant with AA (ANOVA: $P = 0.0001$). However, when the enzyme is preincubated with these fatty acids for 30 s under the same conditions before AA is added, EPA inactivates the enzyme almost completely. HPA, DHA, and AA were weaker as inactivators (Fig. 1B). An ANOVA test showed that the variations among the means were significantly greater than expected by chance with a *P* value of 0.0001.

In the absence of reducing cosubstrate, the inactivation is much more rapid (28). Figure 2 shows that as little as 1 μM AA gave about 50% inactivation of the enzyme after 30 s. HPA, EPA, and DHA gave an even stronger inactivation than did AA. ANOVA test showed that a significant inhibition was obtained, giving a P value of 0.001.

Figure 3 shows how HPA, EPA, and DHA inhibited thromboxane production in isolated human blood platelets in five experiments, calculated as percentage of the formation with AA alone. This was confirmed by a *P* value of 0.0001 by the ANOVA test. All the n-3 fatty acids inhibited in every experiment, but the amounts of thromboxane B_2 formed with AA alone varied from one individual to the other in the range 324–1460 pmoles thromboxane B_2 (3×10^8 platelets).

TABLE 3
Substrate Specificity of Cyclooxygenase and 5-Lipoxygenase[a]

Fatty acid	Cyclooxygenase[b] 30 μM	Cyclooxygenase[b] 60 μM	5-Lipoxygenase[b] 100 μM
20:4	439.6 ± 26.7	563.9 ± 33.5	134–141
20:5	109.5 ± 2.5***	260.2 ± 29.4***	149–145
21:5	27.1 ± 7***	26.6 ± 0	8–7
22:6	28.3 ± 3	42.8 ± 5.4	24

[a]The cyclooxygenase-catalyzed reaction (crude prostaglandin H-synthase) was started by adding fatty acid to a final concentration of 30 or 60 μM, and the oxygen consumption was monitored for 2 min (see Assay Methods section). The results are presented as mean of three replicates ± SD. Crude 5-lipoxygenase (5×10^7 cell equivalents) was incubated as described in the materials and Assay Methods sections. Each of the two replicates is presented in the table. ***$P < 0.001$ vs. 20:4.
[b]O_2 uptake (nmole/min).

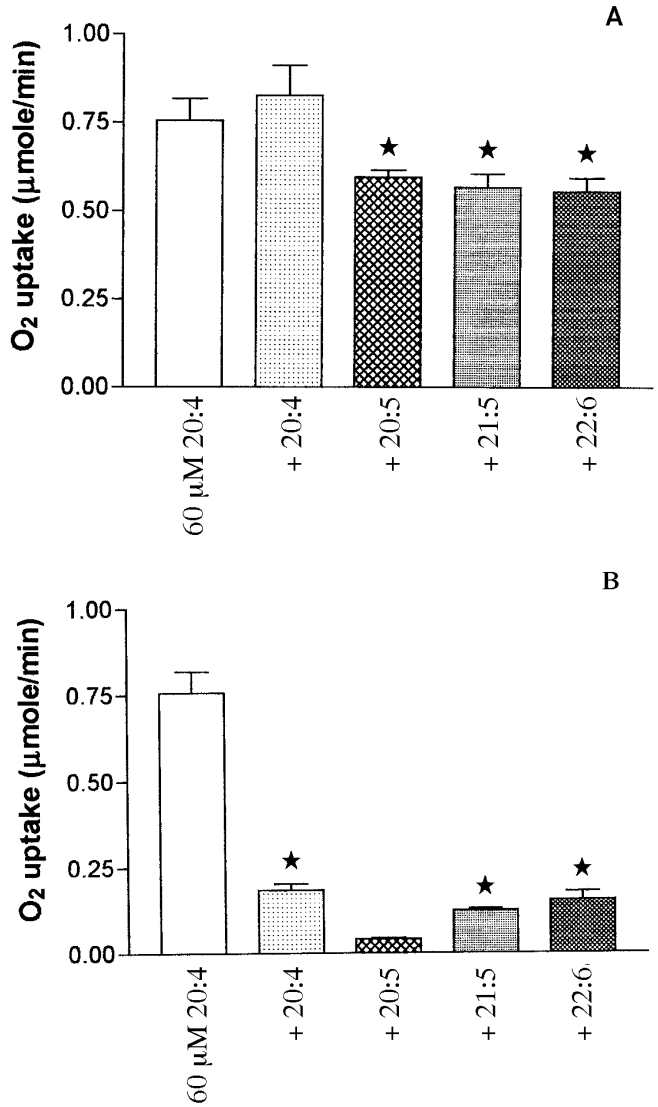


FIG. 1. n-3 Fatty acids as inhibitors/competing substrates of cyclooxygenase, measured as oxygen uptake. Reaction conditions were the same as in Table 2, using crude PGH-synthase as enzyme. A: Arachidonic acid (AA) (20:4n-6) [final concentration (conc.): 60 µM] and n-3 fatty acid (or extra AA), final concentration: 30 µM, were added simultaneously, and initial reaction rate was measured. *: $P < 0.05$ vs. control. B: Cyclooxygenase was preincubated without or with AA or n-3 fatty acid (30 µM, final conc.), 30 s before the addition of AA (final conc.: 60 µM) and measurement of reaction rate; *$P < 0.05$ vs. 20:5. Each bar represents the mean of three replicates ± SD.

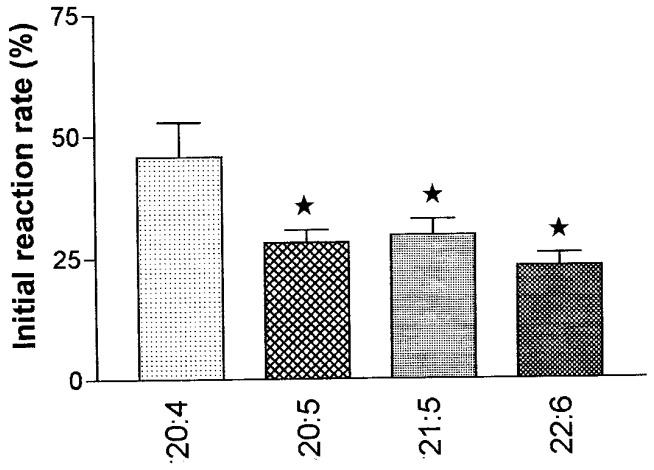


FIG. 2. n-3 Fatty acids and AA as inactivators of PGH-synthase, measured as oxidation of reducing cosubstrate. Crude PGH-synthase (25 µg protein) in 1 mL 0.1 M Tris (pH 8.0) was incubated with 1 µM (final conc.) AA or n-3 fatty acid. After 30 s, AA (final conc. 20 µM), leuco dichlorofluorescein diacetate (1DCF) (final conc. 20 µM) and phenol (final conc. 0.4 mM) were added. Each bar indicates the mean of three replicates ± SD. Results are presented as percentage of the initial change in o.d. at 502 nm with 20 µM (final conc.) AA as substrate. *$P < 0.01$ vs. 20:4. See Figure 1 for abbreviation.

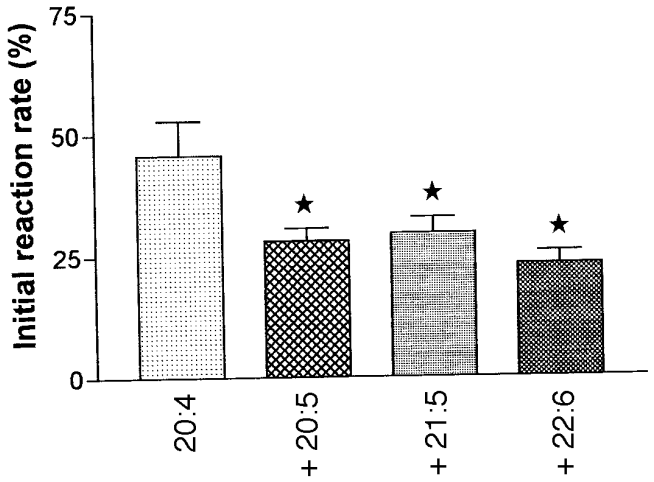


FIG. 3. Inhibition of thromboxane B_2 (TxB_2) production in isolated human blood platelets by preincubation with n-3 polyunsaturated fatty acids. For inhibition studies, 10 µM (final conc.) eicosapentaenoic acid (EPA) (20:5), heneicosapentaenoic acid (HPA) (21:5), or docosahexaenoic acid (DHA) (22:6) were added to the platelet suspension 30 s before addition of AA (final conc.: 10 µM). After 4 min incubation, the production of TxB_2 was estimated by enzyme immunoassay (see assay procedure). In each experiment the amount of TxB_2 produced with AA alone as substrate is set at 100%. Inhibition of TxB_2 production is calculated as percentage of TxB_2 produced with AA. [2–62 pmole TxB_2 was measured in (unstimulated) control platelets (3×10^8)]. Each bar represents the mean of five experiments ± SD. *$P < 0.01$, **$P < 0.001$ vs. 20:4. See Figure 1 for other abbreviation.

5-Lipoxygenase. Table 3 shows, as previously reported (3,29–31), that EPA and AA are about equal as substrates for 5-lipoxygenase, while HPA, like DHA, is a poor substrate. Like PGH-synthase, 5-lipoxygenase is an autoinactivating enzyme (32). We therefore tested whether EPA, HPA, DHA, and AA inactivate 5-lipoxygenase. All these fatty acids inactivated the enzyme only to a moderate extent, both when 5-HETE + 5-HPETE and 5,12-diHETE were measured (not shown).

Induction of acyl-CoA oxidase. Figure 4 shows that acyl-CoA oxidase activity increases threefold in hepatoma cells

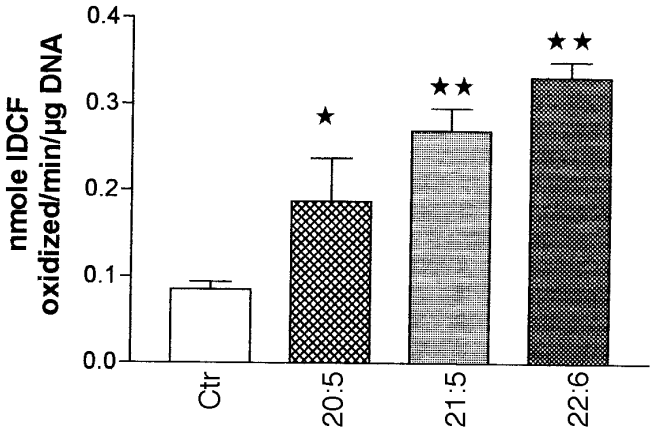


FIG. 4. EPA (20:5), HPA (21:5), and DHA (22:6) as inducers of acyl-CoA oxidase in 7800 C1 hepatoma cells. Confluent hepatoma cells were grown with 80 µM (final conc.) n-3 fatty acids in the medium for 3 d, and the acyl-CoA oxidase was measured with palmitoyl-CoA as substrate (see assay methods). The results are presented as mean of three replicates ± SD. Enzyme activity is expressed as nmoles of oxidized leuco-dichlorofluorescein diacetate (IDCF) per minute and per µg DNA; $^{*}P < 0.05$, $^{**}P < 0.001$ vs. control. See Figures 2 and 3 for abbreviations.

grown for 3 d with 80 µM DHA in the medium. The ANOVA test gave a P value of 0.0001. Student's t-test showed that DHA is a stronger inductor of acyl-CoA oxidase than EPA and HPA ($P < 0.05$), but these acids are all weak inducers compared, e.g., with 3-thia fatty acids (25).

DISCUSSION

Biological effects of fish oils are usually ascribed to their content of EPA and DHA which reduce the content of AA in phospholipids, both by direct incorporation and by inhibition of its synthesis. Since PG and leukotrienes are formed mainly from AA liberated from phospholipids, n-3 fatty acids presumably influence the formation of PG and leukotrienes by inhibiting AA synthesis, by substituting for AA in the phospholipids, and by competing with AA as substrates or inactivators in prostanoid formation.

In the present study we have therefore compared HPA, EPA, and DHA as inhibitors of AA synthesis, as precursors of (phospho)lipids, and as substrates and/or inhibitors of PGH synthase and 5-lipoxygenase, which are key enzymes in the formation of eicosanoids. Previously, HPA has been included only in a study on the substrate specificity of 5-lipoxygenase (3). Our experiments can be seen as a study on the specificity of the different enzyme systems with regard to chain length and position and number of double bonds of the fatty acids. The incorporation of EPA, HPA, and DHA into phospholipids and triglycerides shows that the involved enzymes exhibit low specificity with regard to these fatty acids. They are incorporated equally well, mainly at the expense of oleic acid in hepatocytes. In hepatoma cells they are incorporated at the expense of AA. Our most striking observation is that HPA gave a 90% inhibition of AA synthesis from linoleic acid, whereas AA gave only 50% inhibition, and EPA and DHA showed effects between these.

During conversion of linoleic acid to AA, an increase in dihomo-γ-linolenic acid was observed on the HPLC chromatograms when HPA was used as inhibitor, compared to the other fatty acids (not shown). This indicates that the activity of Δ5 desaturase was inhibited. Analyzing the inhibition of the separate desaturase activities (admittedly under nonidentical conditions), in this conversion of linoleic acid to AA, showed that HPA inhibited Δ5 desaturase strongest whereas EPA was the most potent, but relatively weak inhibitor of the microsomal conversion of linoleic acid to γ-linolenic acid. HPA inhibited the conversion of linoleic acid to AA more efficiently than the desaturation of dihomo-γ-linolenic acid (Table 2, columns 2 and 3). It is a possibility therefore that HPA also inhibits the activity of the elongase which catalyzes the elongation of 18:3 to 20:3. From the results obtained, it looks as if Δ5 desaturase is the main inhibition site for HPA on the conversion of linoleic acid to AA. It is paradoxical that HPA, a fatty acid with a Δ6-double bond, seems more inhibitory on the Δ5 desaturase than AA and EPA which have the first double bond in the Δ5 position. In this connection it is interesting that petroselinic acid (Δ6 monounsaturated octadecaenoic acid) in feeding experiments decreases the level of AA in liver (33), again suggesting an inhibition by the Δ6 double bond on the conversion of linoleic acid.

In yeast, a Δ9 double bond is significant in an inhibition of the Δ9 desaturase (34). Both oxygen uptake and oxidation of reducing cosubstrate show that the PGH-synthase has a high specificity for AA.

According to Evans and Sprecher (35), hydrogen is removed from C_{10} by 12-lipoxygenase and from C_{13} by cyclooxygenase but only in fatty acids where the hydrogen is located at the center of a 1,4-*cis,cis*-pentadiene or a 1,4-pentadiyne system. Hydrogen is removed from C_7 by 5-lipoxygenase. Both AA and EPA have this configuration at C_7 and C_{13}.

However, the introduction of one more double bond in the n-3 position reduces the ability of EPA to be a substrate for PGH-synthase, but not for 5-lipoxygenase. Elongation with one more carbon at the carboxyl end moves the removable hydrogen to the 11- and 14-positions. This combined with the n-3 double bond almost abolished the ability of the acid to be a substrate both for PGH-synthase and for 5-lipoxygenase. Further elongation to DHA made little difference.

However, the preincubation studies on PGH-synthase showed less specificity in the ability of the fatty acids to inactivate this suicidal enzyme. This inactivating effect may be the explanation for the ability of all the n-3 fatty acids to inhibit thromboxane synthesis in platelets (in preincubation experiments). This may contribute to the antithrombotic effect of EPA and DHA, and this effect is probably also shared by HPA.

A discrepancy between the ability to act as substrates and to act as inhibitors is observed also with 5-lipoxygenase. There is evidently a high specificity for chain length and position of double bonds in the reaction. However, all the n-3 fatty acids inhibited the conversion of [1-^{14}C]AA to a simi-

lar extent, suggesting that AA and the n-3 fatty acids all have a similar affinity for the enzyme.

Conclusion. In phospholipid synthesis, the enzymes do not distinguish well between EPA, HPA, and DHA. In AA synthesis, an elongation of EPA to HPA increases the inhibition of the Δ5 desaturase. The ability to act as substrate for PGH-synthase and 5-lipoxygenase is nearly abolished, but the ability to inactivate PGH-synthase is preserved.

ACKNOWLEDGMENTS

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Characterization of an HL-60 Cell Variant Resistant to the Antineoplastic Ether Lipid 1-*O*-Octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine

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ABSTRACT: A resistant cell line (HL-60R) was selected by incubating HL-60 cells with increasing concentrations of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) and used to examine the mechanism of resistance to the antineoplastic ether-linked lipid. The HL-60R cells exhibited a >10-fold increase in resistance when measured by [^{3}H]-thymidine incorporation in comparison to the HL-60 cell line. ET-18-OCH_3 binding occurred at 4°C and was not saturable at the concentrations tested (1–100 μM), indicating that the binding was receptor-independent. At 4°C, association of ET-18-OCH_3 was low for each cell line. At 37°C, uptake in the HL-60 cells was approximately 5-fold greater in comparison to HL-60R cells at each concentration tested. However, when the cellular content of ET-18-OCH_3 was equal, both cell lines experienced similar declines in cell growth. Cellular incorporation of ether lipid was determined using serum-free media and in the presence of serum albumin or lipoproteins. Reduced uptake by the resistant cell line was observed only in the presence of albumin. A greater proportion of ether lipid could be removed from prelabeled HL-60R cells than from HL-60 cells, by an albumin wash procedure, indicating an increased rate of internalization and retention by the sensitive cell line. ET-18-OCH_3 uptake in the HL-60 cell line was also more sensitive to treatment with endocytic (chloroquine, monensin) or metabolic (NaF, KCN) inhibitors. These results suggest that uptake is the principal determinant influencing sensitivity of the resistant cell line and consists of receptor-independent binding followed by internalization. Differential uptake requires the presence of serum albumin and is dependent on the energy-dependent endocytosis of the ether lipid.

Lipids 32, 715–723 (1997).

The ether lipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) is structurally similar to the biologically active lipid, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine). ET-18-OCH_3 has minimal platelet-activating factor activity (1,2). However, it has selective antineoplastic activity both *in vitro* and *in vivo* [for reviews see: (3–5)]. The sensitivity of neoplastic cells to ET-18-OCH_3 is variable and the factors governing susceptibility toward the ether lipids are presently unknown.

Several mechanisms have been proposed that could influence cell sensitivity. These include effects on proliferative signal transduction (6–8) and phospholipid metabolism (9–11). Several studies have demonstrated the ability of ET-18-OCH_3 to inhibit protein kinase C *in vitro* (12–14) and to interfere with protein kinase C-mediated cellular responses (15–17). A recent study by Boggs *et al.* (18) examining the effect of ET-18-OCH_3 on lipid metabolism has reported that the inhibition of phosphatidylcholine synthesis by ET-18-OCH_3 can be overcome by supplementation with lysophosphatidylcholine, thereby overriding the cytotoxic but not the cytostatic activity of ET-18-OCH_3. Therefore, inhibition of phosphatidylcholine synthesis by ET-18-OCH_3 may not be sufficient to account for the full activity exhibited by this compound. The possibility that ET-18-OCH_3 is itself metabolized and serves as a precursor for active metabolites has also been suggested (19). However, more recent studies indicate that ET-18-OCH_3 is resistant to metabolic breakdown (20,21).

Two reports provide evidence that cellular lipid composition may be a contributing factor influencing cell sensitivity toward ET-18-OCH_3. Chabot *et al.* (11) found that increases in the endogenous ether lipid content of K-562 cells by supplementation of culture media with 1-*O*-hexadecyl-*sn*-glycerol sensitizes this cell line to subsequent treatment by ET-18-OCH_3. Similarly, Diomede *et al*. (22) found that an increase in the cholesterol content of HL-60 cells reduced ether lipid uptake and sensitivity.

Cellular uptake represents the initial mechanism which could influence sensitivity (23). Bazill and Dexter (24) suggested that uptake may be influenced by endocytic inhibitors. However, these results are not invariably observed. Fleer *et al.* (25) report little correlation between sensitivity and the rate of uptake of an ether lipid analog (hexadecylphosphocholine) among six different cell lines. Other studies found

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Abbreviations: ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; FBS, fetal bovine serum; HDL, high density lipoproteins; LDL, low density lipoproteins.

no effect of endocytic or metabolic inhibitors on ether lipid uptake (25–27). Consequently, the significance and mechanism of ET-18-OCH_3 uptake remain unclear.

The human leukemic cell line HL-60 is relatively sensitive (28,29) to ET-18-OCH_3. In this study, we report the development and characterization of a resistant variant (HL-60R). The HL-60R subline was developed to investigate potential mechanisms which may confer resistance to ET-18-OCH_3 and to avoid complications that may arise owing to comparisons among different cell lines.

MATERIALS AND METHODS

Materials. HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD). Fetal bovine serum (FBS), RPMI 1640, glutamine, and penicillin/streptomycin were from Gibco (Grand Island, NY). ET-18-OCH_3 was a gift from Medmark Pharma, GmbH (Munich, Germany). 1-*O*-[9,10-^{3}H]octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine ([^{3}H]ET-18-OCH_3, 56 Ci/mmol) was synthesized as previously described (21). 1-(octadecylthio)-2-methoxy-*rac*-glycero-3-phosphocholine and *N*-[3-(octadecylthio)-2-methoxyprop-1-yl]-*rac-N,N*-dimethyl-*N*-(γ-hydroxypropyl) ammonium were provided by Dr. Claude Piantadosi, University of North Carolina (Chapel Hill, NC). ET-18-OCH_3 and related derivatives (10 mg/mL) were stored in ethanol. Chloroquine (100 mM) was prepared fresh in water. Monensin (4 mg/mL) was prepared fresh in ethanol. Sodium fluoride and all solvents were from Fisher Scientific (Fair Lawn, NJ). Silica G thin-layer plates were from Analtech (Newark, NJ). Lipid standards were from Serdary Research Laboratories (Port Huron, MI). [^{3}H]inulin, [^{3}H]thymidine, and Ecolume were from ICN Radiochemicals (Irvine, CA). En^{3}Hance was from New England Nuclear Research Products (Boston, MA). All other reagents were from Sigma (St. Louis, MO).

Cell culture. HL-60 cells were maintained at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mM glutamine. Cell viability was determined by trypan blue exclusion, and cell cytotoxicity was measured by [^{3}H]thymidine incorporation as described (30). The resistant cell line was removed from selection media a minimum of 1 wk prior to experiments.

Lipid analysis and ET-18-OCH_3 metabolism analysis. Lipid extraction was performed on 1–5 × 10^7 cells using a modified Bligh and Dyer procedure (31). Phospholipid mass was determined by the method of Rouser *et al.* (32). Cholesterol content was assessed with the procedure of Rudel and Morris (33). Phospholipid species were fractionated by thin-layer chromatography using Silica Gel G plates in a chloroform/methanol/acetic acid/H_2O (50:25:8:2, by vol) solvent system. Neutral lipids were resolved in a hexane/diethyl ether/formic acid (90:60:4, by vol) system. Phospholipid and neutral lipid species were identified by co-migration with known standards and visualized with iodine vapor. The subclass composition of phosphatidylethanolamine and phosphatidylcholine was determined by high-performance liquid chromatography as described by Chabot *et al.* (11).

For metabolic studies, 5 × 10^7 cells were labeled with 1 μM [^{3}H]ET-18-OCH_3 (0.9 μCi/mL) for 24 h prior to lipid extraction. Cells were then centrifuged at 1200 × *g* and washed three times with phosphate-buffered saline prior to lipid extraction. Radioactive metabolites were separated by thin-layer chromatography as described above. En^{3}Hance was used to visualize radioactive metabolites by autoradiography. Radioactive bands were then scraped into scintillation vials containing 200 mL water. Samples were measured by liquid scintillation counting in 3 mL Ecolume.

ET-18-OCH_3 uptake. The amount of ET-18-OCH_3 uptake is defined and expressed as the amount of cell-associated ET-18-OCH_3 relative to the total cellular phospholipid content (mol%). Unless otherwise indicated, 2 μM ET-18-OCH_3 was used for uptake studies, and incubations were conducted at 37°C for 4 h in 10% FBS. Viability remained above 90% and was not reduced by treatment with ether lipid or inhibitors during this time. ET-18-OCH_3 (10 mg/mL) and radiolabeled ET-18-OCH_3 were dried under nitrogen and resuspended in culture media 1 h prior to use, to obtain a final concentration of 2 μM at 0.1 μCi/mL. Any inhibitors to be used were then added. Cells (1 × 10^7/mL) were added per mL of the assay mixture. In order to determine the amount of cell-associated ET-18-OCH_3, 1-mL aliquots were transferred to Eppendorf centrifuge tubes and centrifuged at 3000 rpm for 2 min in an Eppendorf 5415 C centrifuge (Hamburg, Germany). Cell pellets were washed twice in phosphate-buffered saline (pH 7.4). Samples were then digested overnight in 50 μL 1 M NaOH at room temperature; 50 μL 1 M HCL was added and radioactivity was measured by liquid scintillation counting. For binding experiments, the incubation time was reduced to 1 h in order to maintain cell viability at the higher concentrations, and cells were incubated at 4°C to prevent internalization of ET-18-OCH_3. For experiments under serum-free conditions, it was necessary to reduce the concentration of ET-18-OCH_3 to 0.5 μM to prevent cell lysis. For determination of surface-bound vs. internalized ET-18-OCH_3, cells were prelabeled with ET-18-OCH_3 for 4 h. HL-60 cells were incubated with 1 μM, and HL-60R were incubated with 3 μM ET-18-OCH_3 in order to achieve comparable levels of ether lipid in each cell type. Cells were then pelleted and resuspended in an equal volume of RPMI containing 2.5 mg/mL bovine serum albumin for the times indicated and harvested as described above. This procedure is reported to remove ET-18-OCH_3 from the external surface of the plasma membrane, and the radioactivity remaining represents the amount of internalized ET-18-OCH_3 (34,35).

Lipoproteins. Low density lipoproteins (LDL) and high density lipoproteins (HDL) were purified from human plasma by sequential flotation ultracentrifugation (36) then dialyzed against phosphate-buffered saline. Initially, [^{3}H]ET-18-OCH_3 was added to the isolated LDL and HDL fractions. Labeled HDL and LDL were then repurified by gel filtration chromatography to remove any unbound ET-18-OCH_3. However,

ET-18-OCH_3 absorption to lipoproteins was quantitative, and this repurification step was eliminated in subsequent preparations. LDL, HDL, and albumin concentrations were adjusted to approximate the concentrations found under normal culture conditions of 10% FBS. For uptake experiments, the final concentration of LDL was 22 μg/mL, 5 μg/mL for HDL, 2.5 mg/mL albumin, and 2 μM for ET-18-OCH_3 in RPMI.

Pinocytosis. [^{3}H]inulin was used as a marker for fluid phase endocytosis (37), and experiments were conducted under the same conditions used for ET-18-OCH_3 uptake except for the use of [^{3}H]inulin as a radiotracer. Prior to use, [^{3}H]inulin was purified to remove low molecular weight contaminants by size exclusion column chromatography using a Bio-gel P-10 (70 × 100 mm) column (Bio-Rad, Melville, NY). Pinocytosis was assayed using a final concentration of 2 μM inulin.

RESULTS

Establishment of drug resistance. The HL-60 cell line was cultured at an initial concentration of 0.8 μM ET-18-OCH_3. Cell density was measured every 2 d and the concentration of ET-18-OCH_3 was increased in increments of 0.3 μM when cell growth was observed. After a 3-mon selection process, a resistant variant (HL-60R) was established. The HL-60R cell line was continuously maintained in 4 μM ET-18-OCH_3 (2-fold higher than the ID_{50} for the HL-60 cell line). The level of resistance in the resistant variant was estimated from ID_{50} values derived from [^{3}H]thymidine incorporation studies. The HL-60R cell line exhibited a >10-fold level of increased resistance to ET-18-OCH_3 with respect to the parental HL-60 cell line (Fig. 1). The HL-60R cell line was also resistant to a thioether derivative, 1-(octadecylthio)-2-methoxy-*rac*-glycero-3-phosphocholine but not the nonphosphorus derivative, *N*-[3-(octadecylthio)-2-methoxyprop-1-yl]-*rac*-*N,N*-dimethyl-*N*-(γ-hydroxypropyl) ammonium. The HL-60R cell line was stable and could be removed from selection media for at least 4 mon and still retain its resistance to ET-18-OCH_3. Six subclones were selected from colonies grown in soft agar from the HL-60R cell line. Characterization of each subclone according to its growth rate or in its sensitivity, metabolism, or uptake to ET-18-OCH_3 did not reveal any differences in comparison to the original HL-60R cell line (data not shown). The original HL-60R cell line therefore appeared to be a homogeneous population and was used for subsequent experiments.

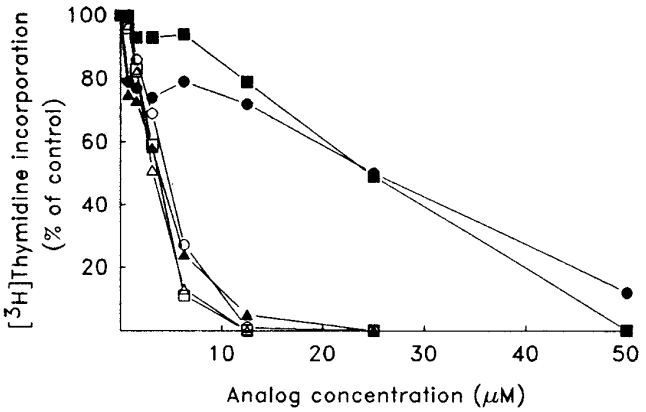

FIG. 1. Effect of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) and related analogs on [^{3}H]thymidine incorporation in the HL-60 (open symbols) and HL-60R (closed symbols) cell lines. Cells were incubated at the indicated concentrations of ET-18-OCH_3 (○,●), 1-*S*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (□,■), or 1-*S*-octadecyl-2-*O*-methyl-*rac*-glycero-3-(*N,N*-dimethylamino) propanol (△,△) for 48 h. [^{3}H]thymidine was then added, and cells were incubated for an additional 8 h. Cells were harvested and radioactivity determined as described (30). Values are presented as a percentage of control values (untreated cells) and are the means of triplicates, SD < 10%. Results shown are one of three similar experiments.

Lipid composition analysis. A comparison of the relative amounts of major phospholipid species or subclass composition (diacyl, alkylacyl, alkenylacyl) of phosphatidylcholine and phosphatidylethanolamine did not reveal significant differences between the two cell lines (data not shown). Cholesterol content was assessed and was slightly reduced in the HL-60R cell line (33.8 ± 2.7 nmoles/10^7 cells, n = 4) cells compared to the HL-60 cell line (43.2 ± 4.0 nmoles/10^7 cells, n = 4). This was reflected in a comparable reduction in total phospholipid content in HL-60R cells (119 ± 11.3 nmoles/10^7 cells, n = 4) compared with the HL-60 cells (143 ± 13.9 nmoles/10^7 cells, n = 4). Consequently, a ratio of phospholipid to cholesterol remained essentially the same for HL-60 (3.31) and HL-60R (3.52).

Metabolism of ET-18-OCH_3. The ability to degrade ET-18-OCH_3 was relatively low for both cell lines. Analysis by thin-layer chromatography showed some increase in the neutral lipid and phospholipid fractions of HL-60R cells within 24 h of incubation. However, the bulk of ET-18-OCH_3 remained in its original form (>90%), and further analysis of metabolites was not undertaken. This is in agreement with previous studies reporting low levels of metabolism for ET-18-OCH_3 (20,21).

Binding and uptake of ET-18-OCH_3. Binding of ET-18-OCH_3 was assayed in the HL-60 cell line in order to determine if ET-18-OCH_3 binding is receptor-mediated. Association of the ether lipid with HL-60 cells remained linear with respect to concentration in the range tested (1–100 μM). These results suggested that binding was not receptor-mediated and was more likely occurring by nonspecific absorption to the plasma membrane (data not shown).

Binding and uptake were subsequently examined in both cell lines during a 6-h period (Fig. 2). At 4°C, ET-18-OCH_3 binding was low and remained relatively unchanged during the time course. Binding equilibrium therefore appeared to be established by the earliest sampling time at 1 h. No difference in binding between the two cell lines was observed. At 37°C, ET-18-OCH_3 uptake increased throughout the duration of the experiment in both cell lines. Under these conditions, a difference in the rate of uptake between the two cell lines was

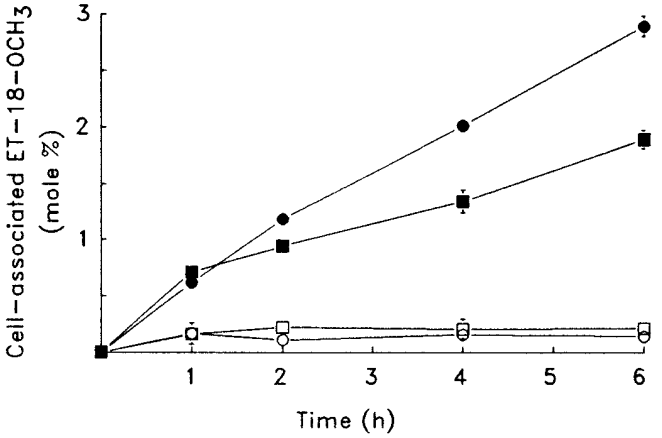

FIG. 2. Time-dependent incorporation of ET-18-OCH_3 in the HL-60 (○,●) and HL-60R (□,■) cell lines. 5×10^5 cells/mL were incubated in presence of 2 μM ET-18-OCH_3 plus 0.1 μCi/mL [^{3}H]ET-18-OCH_3 in 10% fetal bovine serum at 4°C (open symbols) or 37°C (closed symbols). At the indicated times, cell-associated ET-18-OCH_3 was determined as described in the Materials and Methods section and is expressed as the amount of cell-associated ET-18-OCH_3 relative to cellular phospholipid content (mol%). Results are expressed as the means of triplicate samples ± SD and are one of three similar experiments. See Figure 1 for abbreviation.

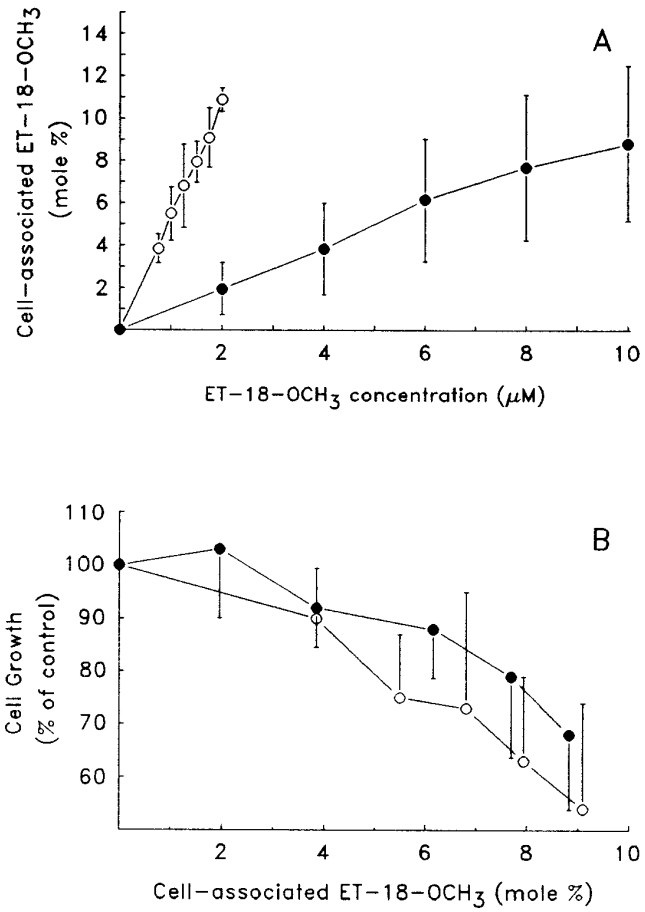

FIG. 3. Long-term uptake of ET-18-OCH_3 and its effect on cell growth in the HL-60 and HL-60R cell lines. (A) Concentration-dependent uptake of ET-18-OCH_3, (B) ET-18-OCH_3 content vs. growth inhibition. The HL-60 (○) and HL-60R (●) cell lines were cultured at an initial density of 5×10^5 cells/mL at the indicated concentrations of ET-18-OCH_3 for 48 h. ET-18-OCH_3 uptake was then determined as described in the Materials and Methods section, and viable cell counts were measured by trypan blue exclusion. Cell growth is represented as a percentage of control values (untreated cells). ET-18-OCH_3 uptake is represented as a mol% of the total phospholipid content. Results are expressed as the means ± SD of five experiments performed in triplicate. See Figure 1 for abbreviation.

observed and clearly evident by 4 h. At this time, uptake was reduced in the HL-60R cell line by approximately 65% relative to the HL-60 cell line. A correlation between uptake and sensitivity was indicated by these results. However, no equilibrium was evident within this time period, and a more detailed examination of ET-18-OCH_3 uptake was therefore undertaken.

ET-18-OCH_3 content and cell growth. ET-18-OCH_3 uptake was next examined in the two cell lines during 2-d incubations at multiple concentrations of ET-18-OCH_3. At the end of the incubation time, ET-18-OCH_3 content was assessed and compared to effects on cell growth. From these results, the extended incubation time increased the differences in uptake previously seen. The difference in uptake between the two cell lines at a given concentration was approximately 5-fold (Fig. 3A). Comparison of ET-18-OCH_3 content vs. cell growth (Fig. 3B) demonstrated similar declines in cell growth in both cell lines. These results indicate that reduced uptake was responsible for the resistance observed in the HL-60R variant since the growth of both cell lines deceased when the ET-18-OCH_3 content was comparable.

Role of serum in ET-18-OCH_3 uptake. The uptake of ET-18-OCH_3 under serum-free conditions or in the presence of individual serum components was next examined. Under serum-free conditions, uptake was considerably higher and 2 μM ET-18-OCH_3 resulted in reduced cell viability. Therefore, 0.5 μM was subsequently used during these experiments. Uptake was equal between the two cell lines in the absence of serum, and uptake was no longer temperature-dependent (Table 1). These results indicated a requirement for serum in mediating the differential uptake of ET-18-OCH_3 in the HL-60 and HL-60R cell lines. Therefore, the uptake of ET-18-OCH_3 in the presence of individual serum components was investigated.

Substantial uptake of ET-18-OCH_3 in association with LDL or HDL could be seen and was not reduced in the HL-60R cell line (Fig. 4). The comparable uptake of ET-18-OCH_3/LDL suggested the two cells lines possessed equal abilities in receptor-mediated endocytosis. These results further indicate that lipoproteins may contribute to the total uptake of ET-18-OCH_3 but do not appear responsible for the difference in uptake seen between the HL-60 and HL-60R cell lines. In these experiments, the final concentration of LDL was 22 μg/mL or 5 μg/mL for HDL, and 2 μM ET-18-OCH_3. In the presence of complete serum, 2 μM ET-18-OCH_3 is distributed among several serum components (38). Consequently, the ET-18-OCH_3 content of LDL and HDL is higher

TABLE 1
Cellular Association of ET-18-OCH_3 Under Serum-Free Conditions[a]

Temperature	HL-60 Uptake (mol%)	HL-60R Uptake (mol%)
4°C	4.49 ± 0.561	5.66 ± 0.277
37°C	5.18 ± 0.223	6.09 ± 0.771
% Reduction	13%	7%

[a]The association of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycerol-3-phosphocholine (ET-18-OCH_3) (0.5 μM) with the HL-60 or HL-60R cell lines was measured in the absence of serum either at 37°C or at 4°C for 1 h using 5×10^5 cells/mL as described in the Materials and Methods section. Results are expressed as the amount of ET-18-OCH_3 associated with cells relative to total phospholipid content (mol%). Results are presented as the means of triplicate samples ± SD and representative of three experiments.

in these experiments in comparison to the fraction of 2 μM ET-18-OCH_3 normally associated with these lipoproteins in complete serum. Therefore the contribution of these lipoproteins in ET-18-OCH_3 uptake is likely to be exaggerated under these conditions. Reduced uptake by the HL-60R cell line was observed only when albumin or serum was present (Fig. 4).

Albumin appeared important in mediating differential uptake, and ET-18-OCH_3 uptake was consequently measured in the presence of increasing concentrations of albumin (1–10 mg/mL). Uptake was effectively reduced by increasing albumin concentration and was lower in the HL-60R cell line in comparison to the HL-60 cell line at all concentrations tested. Increasing the concentration of albumin presumably alters the partitioning equilibrium thereby increasing the back exchange of ET-18-OCH_3 from cells to albumin. Consequently, whether an ET-18-OCH_3/albumin complex was binding could not be determined from this experiment. However, the nonsaturability of ET-18-OCH_3 binding at increasing ether lipid concentration at 4°C discounts this possibility.

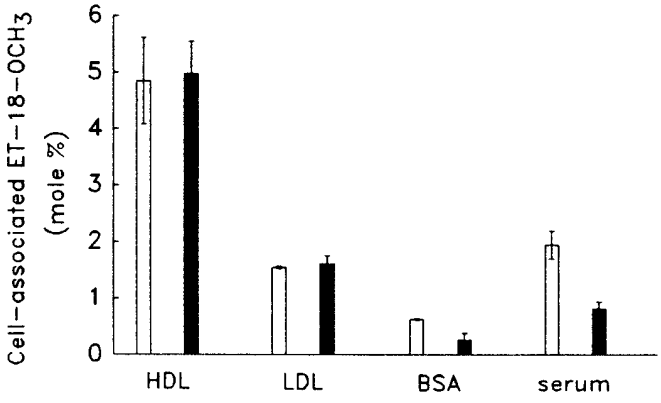

FIG. 4. ET-18-OCH_3 uptake in the presence of individual serum components. Uptake in the HL-60 (open bars) or HL-60R (closed bars) cell lines was measured in the presence of 5 μg/mL high density lipoprotein (HDL), 22 μg/mL low density lipoprotein (LDL), 2.5 mg/mL bovine serum albumin, or 10% fetal bovine serum plus 2 μM ET-18-OCH_3. ET-18-OCH_3 uptake was determined as described in the Materials and Methods section and is expressed as the amount of cell-associated ET-18-OCH_3 relative to cellular phospholipid content (mol%). Results are expressed as the means of triplicate samples ± SD and are one of three similar experiments. BSA, bovine serum albumin. See Figure 1 for other abbreviation.

To confirm that the cellular association of ET-18-OCH_3 was rate-limiting and not dissociation of ET-18-OCH_3 from albumin, uptake was measured as a function of increasing cell density (1 to 20×10^5/mL). Increasing cell density should increase the frequency of ET-18-OCH_3/cell interactions and enhance uptake if binding is rate-limiting. Increasing cell density should not affect uptake if dissociation from albumin is rate-limiting. ET-18-OCH_3 uptake in the presence of albumin was indeed proportional to cell number and similar results were obtained using 10% FBS (data not shown). Based on these results, albumin appears to serve as a reservoir from which ET-18-OCH_3 can rapidly transfer to the cell membrane.

Inhibition of ET-18-OCH_3 uptake. Bazill and Dexter (24) have shown that endocytosis may represent at least one component of ET-18-OCH_3 uptake and may distinguish ET-18-OCH_3-sensitive cells from more resistant cell lines. Therefore, we examined the proportion of uptake attributed to endocytosis in each of our cell lines through the use of the endocytic inhibitors, chloroquine (39,40) and monensin (41) or the metabolic inhibitors, NaF and KCN. The HL-60 cell line was most sensitive to treatment with these agents, and ET-18-OCH_3 uptake was reduced in response to all the compounds tested (data not shown). Monensin was the most effective inhibitor tested, and reductions in uptake were observed in both cell lines. However, the HL-60 cell line was most affected by monensin treatment. The lowest concentration of monensin (12.5 μM) reduced the level of uptake in the HL-60 cell line to a level comparable to untreated HL-60R cells. Further reductions in ET-18-OCH_3 uptake were not observed above 50 μM monensin.

Assessment of internal vs. external ET-18-OCH_3. The results obtained with the inhibitors above suggested that an endocytic mechanism was an important feature in distinguishing ET-18-OCH_3-sensitive from -resistant cell lines. These results were further supported by experiments measuring internalized ET-18-OCH_3 following removal of externally bound ether lipid. In these experiments, cells were incubated for 4 h in 1 μM (HL-60) or 3 μM ET-18-OCH_3 (HL-60R) in order to obtain approximately equal amounts of ET-18-OCH_3 in either cell line. The ET-18-OCH_3 bound to the outer surface of the plasma membrane was then removed by washing cells with RPMI + 2.5 mg/mL bovine serum albumin, and the amount remaining represented internalized ET-18-OCH_3. At 4°C, this procedure resulted in removal of approximately 46% of the ET-18-OCH_3 in HL-60R but only 12% from HL-60 cells (Fig. 5). At 37°C, a gradual loss of ET-18-OCH_3 over 2 h could be observed and appeared comparable between the two cell lines. This decline resulted in nearly complete elimination of cell-associated ET-18-OCH_3 in the HL-60R cell line by the end of the incubation.

Pinocytic uptake. Fluid phase endocytosis was measured using [^{3}H]inulin as a marker to obtain a general measure of endocytic activity in either cell line. Both cell lines endocytosed this compound in a time-dependent manner (Fig. 6). However, reduced uptake was not observed in the resistant

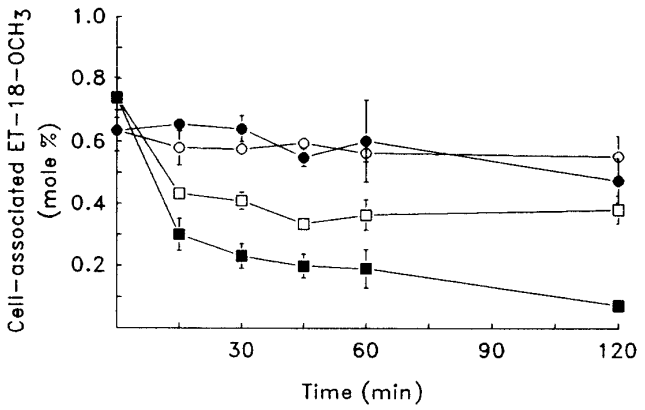

FIG. 5. Removal of externally bound ET-18-OCH_3 from HL-60 (○,●) and HL-60R (□,■) cells which were prelabeled by incubation for 4 h with 1 μM or 3 μM ET-18-OCH_3, respectively. This procedure provided comparable levels of ET-18-OCH_3 between the two cell lines. Cells were pelleted then resuspended (5×10^5/mL) in media containing 2.5 mg/mL bovine serum albumin and incubated at 4°C (open symbols) or 37°C (closed symbols). One-mL aliquots were sampled at the indicated times. Cell-associated ET-18-OCH_3 was determined as described in the Materials and Methods section and is expressed as the amount of cell-associated ET-18-OCH_3 relative to cellular phospholipid content (mol%). Results are expressed as the means of triplicate samples ± SD and are one of three similar experiments. See Figure 1 for abbreviation.

cell line. Similar results have been observed by Sleight and Abanto (34) who have reported that there was little agreement between the pinocytic rate of uptake of horseradish peroxidase and the differences observed in the uptake of lipids among different cell lines. Fluid-phase pinocytosis occurs at a lower magnitude in comparison to substances which can bind to the surface of a cell. The uptake of endocytic markers absorbed to the plasma membrane can be 100 to 1000 times the rate of uptake of a fluid phase endocytic marker (42).

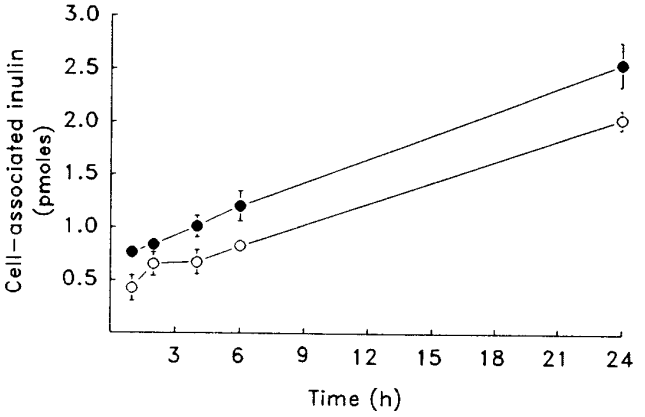

FIG. 6. Time-dependent uptake of [^{3}H]inulin in the HL-60 (○) and HL-60R (●) cell lines. 5×10^5 cells/mL were incubated in the presence of 2 μM inulin (0.5 μCi/nmole) in culture media at 37°C for the indicated times. Inulin uptake was then measured as described in the Materials and Methods section. Results are expressed as pmoles of cell-associated inulin per 5×10^5 cells. Results are the means of triplicate samples ± SD and are one of three similar experiments.

Therefore, measurements of fluid-phase endocytosis may be limited in revealing differences in absorptive endocytosis.

DISCUSSION

In this study, we report the development and characterization of an HL-60 variant (HL-60R) displaying increased resistance to the antineoplastic ether lipid, ET-18-OCH_3. Reduced uptake of ET-18-OCH_3 by the HL-60R cell line was observed during incubations up to 6 h. Long-term incubations (2 d) enhanced these differences. A comparison of ET-18-OCH_3 content and cell growth revealed that both cell lines experienced similar declines in cell growth when the ET-18-OCH_3 content between the two cell lines was equal. This result provided evidence that uptake was the primary difference determining ET-18-OCH_3 sensitivity between these two cell lines.

Binding of ET-18-OCH_3 to HL-60 cells was concentration-dependent and was not saturable in the range tested (1–100 μM). This lack of saturability indicates that ET-18-OCH_3 does not bind through a specific receptor interaction. It remains possible that this compound could be absorbed into the plasma membrane and subsequently interact with a transport protein. However, the existence of a phosphatidylcholine or lysophospholipid transporter accessible to the external plasma membrane has not been established. In addition, the sensitivity of ET-18-OCH_3 uptake to multiple inhibitors in our studies argues against this possibility.

Kelley *et al.* (27) examined uptake under serum-free conditions in the L1210 cell line and concluded that uptake occurred by passive diffusion since uptake could not be reduced by endocytic or metabolic inhibitors. We examined uptake under serum-free conditions using the HL-60 and HL-60R variant as well. These results were at marked contrast with those observed in the presence of serum. Uptake was no longer reduced in the resistant cell line or temperature-dependent. These results suggested that uptake under serum-free conditions was not representative of physiological conditions and is instead, perhaps, a measure of ET-18-OCH_3 partitioning between the media and cells. Passive diffusion did not appear to be responsible for the differential uptake of ET-18-OCH_3 in our cell model since uptake responded to endocytic and metabolic inhibitors. In addition, diffusion as a predominant mechanism of ET-18-OCH_3 uptake seems unlikely since transbilayer movement of lysophosphatidylcholine is reported to be extremely low owing to the polar headgroup of this compound (43).

A study by Bazill and Dexter (24) demonstrated that ET-18-OCH_3 uptake could be reduced by endocytic or metabolic inhibitors in the murine myelomonocytic leukemic WEHI-3B cell line and in the multipotential nonleukemic FDCP-mix A4 cell line. They concluded that endocytosis is the principal determinant distinguishing sensitive cell lines from more resistant cell lines. In a related study, Storch and Munder (26) also reported reduced uptake in a fibrosarcoma-resistant cell line but were unable to suppress uptake using NaF as a metabolic inhibitor. The discrepancy between this study and the work

of Bazill and Dexter (24) suggests that NaF-sensitive endocytosis of ET-18-OCH_3 may be a cell type-specific phenomenon. Our results support an active, endocytic uptake of ET-18-OCH_3 as the determining factor between the sensitive and resistant cell lines. Treatment of HL-60 cells with endocytic inhibitors, chloroquine or monensin, or the metabolic inhibitors, NaF or KCN, suppressed ET-18-OCH_3 uptake. The resistant cell line was less sensitive to these treatments. Monensin was most effective, and inhibition of ET-18-OCH_3 uptake was examined as a function of increasing monensin concentration. At the lowest concentration tested (12.5 μM), uptake in the HL-60 cell line was reduced to approximately the level of uptake seen in untreated HL-60R cells. Further reductions in uptake were not observed above 50 μM monensin, but the level of uptake did not approach zero even after accounting for binding based on data obtained at 4°C. This remaining level of cell-associated ET-18-OCH_3 was equivalent in the HL-60 and HL-60R cell lines. This residual level could represent either an additional mechanism of uptake not inhibitable by monensin, or an enhanced level of ether lipid binding at 37°C due to increased membrane fluidity at the higher temperature. If spontaneous insertion of ET-18-OCH_3 is temperature-dependent, then binding at 37°C could represent a significant component of total uptake under short incubation times. The effects of inhibitors would be underestimated under these conditions and may partly explain the conflicting results from different studies. In addition, multiple endocytic pathways have been described which respond differently to various inhibitory agents (44–46). ET-18-OCH_3 may enter a cell by any endocytic process. Which pathway predominates is likely to vary among different cell types, and consequently, cell responses to different types of inhibitory compounds may also differ.

The presence of serum was required in order to see differential uptake between the HL-60 and HL-60R cell lines. Therefore, some constituent within serum mediated ET-18-OCH_3 interaction with the cell and uptake was therefore examined in the presence of individual serum components. Lipoproteins are known to transport lipids to cells and were therefore considered as potential carriers of ET-18-OCH_3. Uptake of ET-18-OCH_3 in the presence of both LDL and HDL readily occurred. However, reduced uptake by the resistant cell line was not seen. Both cell lines appeared equal in their capacity of receptor-mediated endocytosis of LDL. Studies characterizing the partitioning of ET-18-OCH_3 among serum components indicate that the majority of ET-18-OCH_3 associates with serum albumin (27,38). Our results are consistent with these observations and suggest serum albumin plays an important role in ET-18-OCH_3 uptake. Uptake of ET-18-OCH_3 in the presence of albumin obeyed the same relationship seen with serum. In the presence of albumin, ET-18-OCH_3 was reduced in the resistant variant in comparison to the HL-60 cell line. Removal of externally bound ET-18-OCH_3 by an albumin wash procedure from prelabeled cells was used as an alternative approach to confirm that differences in endocytosis existed between the HL-60 and HL-60R cell lines. A greater proportion of ether lipid could be removed from the HL-60R cell line, indicating the resistant cell line had internalized less ET-18-OCH_3.

Cell lines that exhibit a multidrug-resistant phenotype commonly have enhanced endocytic rates (47,48). Such cells may consequently have increased sensitivity toward ET-18-OCH_3. Therefore, this compound could provide an alternative treatment for neoplastic cells which are refractory toward other antineoplastic agents. Alternatively, multidrug-resistant cells may also have a higher efflux rate of ET-18-OCH_3 which could result in lower accumulation and resistance to growth inhibition. ET-18-OCH_3 has also shown promise in bone marrow purging experiments (49,50). Our results suggest that the use of individual serum components could increase the efficacy or alter the cellular selectivity of this procedure. The use of specific serum components could be used to exploit individual differences among neoplastic cells.

ACKNOWLEDGMENTS

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Conjugated Linoleic Acid Modulation of Phorbol Ester-Induced Events in Murine Keratinocytes

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ABSTRACT: Recent work in our lab has shown that the chemoprotective fatty acid, conjugated linoleic acid (CLA), inhibits phorbol ester skin tumor promotion in mice. Because little is known about the deposition of CLA into tissues as well as its biological activity, this study compared the incorporation and biological activity of CLA to linoleic acid (LA; 18:2, *c*9,*c*12) and arachidonic acid (AA; 20:4 *c*5,*c*8,*c*11,*c*14) in cultured keratinocytes. When keratinocytes (HEL-30) were grown in media containing ^{14}C-CLA for various periods, more than 50% of the ^{14}C-CLA was incorporated into cellular lipids by 9 h. The distribution of CLA in phospholipid classes was similar to LA. Approximately 50% of ^{14}C-LA and ^{14}C-CLA were incorporated into phosphatidylcholine (PC), while the remainder was taken up by phosphatidylethanolamine (PE) and phosphatidylserine/phosphatidylinositol (PS/PI). In contrast, ^{14}C-AA was more equitably distributed into PC, PE, or PS/PI (27, 30, or 38%, respectively). When keratinocytes were prelabeled with radiolabeled fatty acids, phorbol ester-induced release of ^{14}C-CLA was 1.5 times higher than ^{14}C-LA and ^{14}C-AA. However, ^{14}C-prostaglandin E (PGE) release in ^{14}C-CLA prelabeled cultures was 6 and 13 times lower than cultures treated with ^{14}C-LA and ^{14}C-AA, respectively. Moreover, the ability of nonradiolabeled CLA to support ornithine decarboxylase activity, a hallmark event of tumor promotion, was significantly lower than in LA- and AA-treated cultures. These studies suggest that CLA inhibits skin tumor promotion, in part, through a PGE-dependent mechanism.

Lipids 32, 725–730 (1997).

The term "conjugated linoleic acid (CLA;18:2)" refers to a group of positional and geometric isomeric products derived from the essential fatty acid, linoleic acid (LA; 18:2; *c*9,*c*12). The double bonds of CLA can be at the 9, 11 or 10, 12 positions and in *cis* and/or *trans* spatial configurations. In foods, the principal dietary sources of CLA are cooked meats and pasteurized milk and cheese products (1). While CLA is a polyunsaturated fatty acid found naturally in the food supply, its conjugated dienoic double-bond nature lends unique pluripotent chemopreventive effects in rat mammary (2,3) and colon carcinogenesis (4) as well as mouse forestomach (5) and skin carcinogenesis (6,7). Presently, the physiological and biochemical mechanisms of CLA chemoprotection in multiple tissues are poorly understood (8).

Recently our lab found that dietary CLA inhibited phorbol ester-induced tumor promotion in the mouse skin multistage carcinogenesis model (7). This model of carcinogenesis allows for the stages of initiation, promotion, and progression to be operationally separated (9). In this study, a semipurified diet containing no CLA was fed to mice during initiation with dimethylbenz(a)anthracene. After carcinogen initiation, mice were switched to one of four experimental diets containing incremental levels of CLA from 0.0 to 1.5% (by weight of diets), and mice were topically promoted with the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). These studies clearly demonstrated that, by 25 wk, dietary CLA inhibited TPA-induced tumor promotion in a manner that was independent of a previously established antiinitiator activity (6,7).

Topical application of TPA to mouse skin results in a variety of morphological and biochemical changes in epidermis, including increased prostaglandin (PG) synthesis, induction of ornithine decarboxylase (ODC) and phospholipase activities, interaction with protein kinase C (PKC) and PKC-dependent gene expression (*via* TPA-responsive elements) as well as papilloma formation (10,11). Over the past few years, the roles of polyunsaturated fatty acids [such as LA, arachidonic acid (AA; 20:4, *c*5,*c*8,*c*11,*c*14) and eicosapentaenoate (20:5, *c*5,*c*8,*c*11,*c*14,*c*17] in mouse skin tumor promotion by TPA have been elucidated (12–14). These studies demonstrated that the multistage skin tumorigenesis model displays a lack of response to n-3 dietary fatty acids and a tumor-inhibiting response to LA (reviewed in Ref. 15). In contrast, other studies have demonstrated n-3 fatty acids inhibit carcinogenesis while the n-6 fatty acid, LA, enhances tumorigenesis in rodent mammary and colon cancers. Compared to the effects of n-3 and n-6 fatty acids, CLA appears to lack organ specificity for its chemopreventive properties. Studies thus far have demonstrated CLA inhibits tumorigenesis in mammary, forestomach, and colon and skin models of carcinogenesis (15).

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Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; CLA, conjugated linoleic acid; LA, linoleic acid; ODC, ornithine decarboxylase; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PGE, prostaglandin E; PI, phosphatidylinositol; PS, phosphatidylserine; TLC, thin-layer chromatography; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Because CLA appears to have such unique pluripotent chemoprotective effects, we are interested in elucidating the ability of CLA to be deposited into tissues and exhibit subsequent biological activity in the regulation of cellular processes associated with lipid homeostasis and tumor promotion. Because LA can serve as the precursor to CLA in nonenzymatic conversion in foods or be converted to AA *via* desaturation and elongation enzymatic conversion in the liver *in vivo* (16), the goal of the present study was to compare the incorporation and biological activity of each of these fatty acids in keratinocytes. In these studies, the keratinocyte cell line HEL-30 was prelabeled with LA, CLA, or AA, then treated with the tumor promoter, TPA, to induce prostaglandin E (PGE) synthesis and ODC activity, two events strongly linked with skin tumor promotion.

MATERIALS AND METHODS

Chemicals. TPA was purchased form LC Laboratories (Woburn, MA) and diluted in acetone. Radiolabeled (1-^{14}C)-LA, (1-^{14}C)-CLA (specific activities, 55 mCi/mmol) were obtained from American Radiolabeled Chemical Inc. (St. Louis, MO). (1-^{14}C)-AA (specific activity, 56 mCi/mmol) was purchased from Moraver Biochemical Inc. (Brea, CA) and (1-^{14}C) ornithine hydrochloride (specific activities, 50–62 mCi/mmol) from Amersham Life Science (Arlington, IL). Nonradiolabeled fatty acids were purchased from Nu-Chek-Prep (Elysian, MN), and the approximate isomeric composition of CLA (98% pure) was 41.2% *c*9,*t*11- and *t*9,*c*11-CLA, 44.1% *t*10,*c*12-CLA, 9.4% *c*10,*c*12, 1.3% *t*9,*t*11, *t*10,*t*12-CLA, 1.1% *c*9,*c*11-CLA, 0.7% LA, and 2.2% unidentified compound. Standards for triglycerides and phospholipids [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS)] were purchased from Sigma Chemical Company (St. Louis, MO) and authentic PGE_2 standard from Cayman Chemical Co. (Ann Arbor, MI). DC-Alufolien Kiesegel 60 F_{254} thin-layer chromatography (TLC) plates were purchased from EM Separation Technology (Gibbtown, NJ).

Cell culture. The murine epidermal cell line HEL-30 (17) (passage levels between 30 and 40) was maintained in Hanks' Balanced Salt Solution containing vitamin and amino acid mixtures supplemented with 100 Units/mL penicillin and 100 µg/mL streptomycin as well as 5% fetal bovine serum. Cultures were incubated at 37°C in a humidified atmosphere of air/CO_2 95:5 (mol%). For experiments, cells were plated at densities of 4×10^5 per 35-mm dish or 6×10^5 per 60-mm dish. The growth medium was a complete medium with serum, and the cultures were incubated until 90% confluence was reached.

Cellular uptake of ^{14}C-fatty acid. After cells were confluent, cultures (35-mm dishes) were labeled with 0.1 µCi/mL ^{14}C-LA, ^{14}C-CLA, or ^{14}C-AA mixed with serum-free media containing 0.1% bovine serum albumin (BSA). At various time points (0–24 h), the labeled media were aspirated from dishes, and cells were rinsed with phosphate-buffered saline (PBS), scraped into methanol (1.0 mL), and counted by liquid scintillation.

Analysis of ^{14}C-fatty acid distribution among phospholipid and triglyceride lipid fractions. Media containing ^{14}C-LA, ^{14}C-CLA, or ^{14}C-AA 0.1 µCi/mL were added to confluent cultures which were then incubated for 12 h. The medium was aspirated from dishes, and cultures were washed once with PBS and frozen (−70°C). Thawed cells were scraped into methanol (1.0 mL) and dishes rinsed by methanol (1.0 mL). Lipids were extracted by adding chloroform and 2 M KCl (3:1). Extracts were dried under nitrogen and resuspended in 150 µL chloroform/methanol (2:1, vol/vol). Phospholipid and triglyceride fractions were separated by normal-phase TLC (chloroform/methanol/acetic acid; 65:45:4, by vol) (13). Bands which comigrated with PC, PS/PI, PE, and triglyceride authentic standards were identified by iodine vapors and scraped from plates into vials for counting by liquid scintillation.

TPA-stimulated fatty acid release and PGE synthesis. Confluent cultures were prelabeled with ^{14}C-LA, ^{14}C-CLA, or ^{14}C-AA at 0.2 µCi per mL of serum-free media with 0.1% BSA. After 12 h incubation, the labeled medium was removed, and cells were washed once with PBS. Acetone (1 µg/mL) or TPA (1.6 µM) was added to media that were then held for 3 h, at which time an aliquot of the medium was quantitated by liquid scintillation. The medium was collected and acidified (pH 3.0) with 1 N HCl, and eicosanoids were extracted twice by ethyl acetate. Eicosanoid extracts were dried under nitrogen, redissolved in ethyl acetate, and applied onto normal-phase TLC plates. Prostaglandins were separated with the organic phase of ethyl acetate/isooctane/glacial acetic acid/water (55:25:10:50, by vol) (13). Iodine vapors were used to identify the position of PGE_2 compared with authentic standards, and the band which comigrated with PGE_2 standard was cut from plates and counted by liquid scintillation.

Ornithine decarboxylase activity. Cells were grown in serum-rich media until confluent. The medium was changed to serum-free medium with 0.1% BSA containing unlabeled LA, CLA, or AA (5 µg/mL medium) for 12 h followed by treatment with serum-free media with 0.1% BSA containing acetone (1 µL/mL) or TPA (1.6 µM) for 6 h; cultures were then washed once with cold PBS and frozen at −70°C until analyses were performed (13). Cells were scraped into ODC buffer containing sodium-potassium phosphate, pyridoxal phosphate, sodium ethylenediaminetetraacetic acid, and dithiothreiotol and homogenized. Homogenates were centrifuged at $12{,}000 \times g$, and 500 µL supernatant was used to determine the ODC activity as we have described previously (14). Protein values of cell homogenates were determined by Coomassie blue reaction (Bio-Rad, Hercules, CA), and ODC activity was expressed as nmol $^{14}CO_2$ released per mg protein per h.

Statistical analysis. Data were analyzed by one-way ANOVA and the Student's *t*-test ($\alpha = 0.05$) with Minitab Statistical Software (Minitab Inc., State College, PA).

RESULTS

Incorporation of ^{14}C-LA, ^{14}C-CLA, or ^{14}C-AA into total cellular lipids. Confluent HEL-30 keratinocytes exhibited maximal cellular uptake of fatty acids after 9 h incubation (Fig. 1). There was not a significant change after 6, 9, 12, 15, and 24 h incubation for any of these fatty acids ($P > 0.05$). More than 80% of total ^{14}C-LA and ^{14}C-AA were incorporated into HEL-30 cells after 12 h incubation, but only 70% of total ^{14}C-CLA was taken up by HEL-30 cells by that same time.

^{14}C-fatty acid distribution among phospholipids and triglycerides. Membrane phospholipid fatty acids serve as the substrate pool for eicosanoid metabolism. Therefore, it was important to compare the extent of fatty acid incorporation into phospholipid classes and triglycerides. Approximately 95% ^{14}C-fatty acids were incorporated into total phospholipids and nearly 5% ^{14}C-fatty acids into triglycerides (Fig. 2). In addition, LA, CLA, and AA were distributed differently among the phospholipid classes. Of the phospholipid-associated fatty acids, 54% of ^{14}C-LA was incorporated into PC, and 23 and 20% were in PS/PI or PE. ^{14}C-CLA demonstrated similar patterns of incorporation into PC, PS/PI, and PE (55, 27, and 14%, respectively). In contrast, ^{14}C-AA was more evenly incorporated into PC, PS/PI, and PE (27, 30, and 38%, respectively).

TPA-stimulated fatty acid release and PGE synthesis. Numerous previous studies have demonstrated that the AA-derived PGE_2 modulates tumor promotion-associated events such as ODC activity, vascular permeability, and hyperplasia (9,10). One of the rate-limiting steps of AA conversion to PGE_2 is the supply of free AA released from phospholipids. TPA-induced free ^{14}C-fatty acid release and PGE synthesis

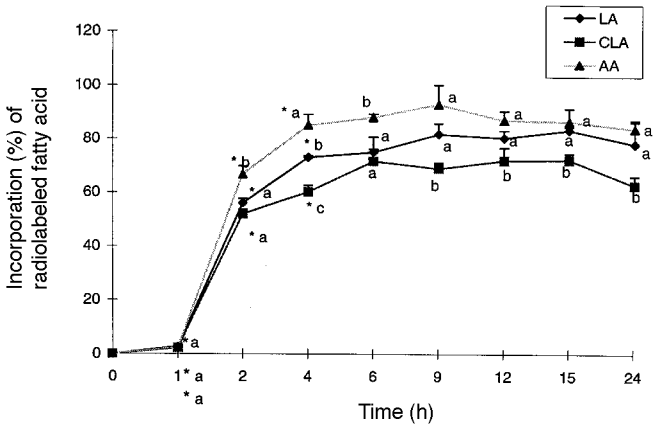

FIG. 1. The percentage of ^{14}C-LA (linoleic acid), ^{14}C-CLA (conjugated linoleic acid), or ^{14}C-AA (arachidonic acid) incorporated into total cellular lipids of HEL-30 keratinocytes. After the removal of media, cells were scraped into methanol and counted by liquid scintillation. Each time point per dish ($n = 3$) represents the mean of percentage incorporation of radiolabeled fatty acid ± SEM from three independent experiments. An asterisk (*) indicates a significant difference between means of each time point and 12 h ($P < 0.05$). Within the same time point, different letters represent a significant difference ($P < 0.05$).

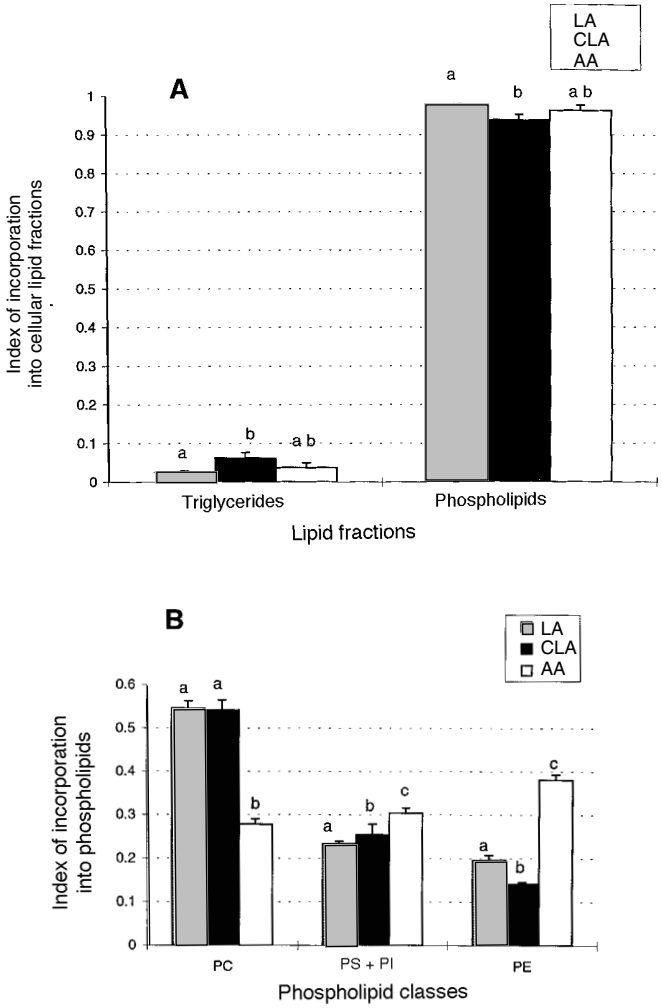

FIG. 2. ^{14}C-Fatty acid distribution among cellular phospholipids and triglycerides of HEL-30 cells. (A) Keratinocyte cultures were prelabeled with 0.1 μCi/mL ^{14}C-LA, ^{14}C-CLA, or ^{14}C-AA and incubated for 12 h. Phospholipids and triglycerides were separated by thin-layer chromatography (TLC) and counted. (B) Phospholipid classes were separated by TLC and data represent mean index of incorporation of total cellular lipid fractions from at least three independent experiments containing triplicate samples ($n = 3$). Within the same lipid fraction, the bar not sharing the same letter was significantly different ($P < 0.05$); PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine. See Figure 1 for other abbreviations.

were significantly greater than acetone treatment. TPA-induced release of ^{14}C-fatty acid could be ranked in decreasing order (^{14}C-CLA > ^{14}C-AA ≅ ^{14}C-LA) (Fig. 3). TPA-induced PGE_2 synthesis in ^{14}C-AA pretreated group was two times higher than in ^{14}C-LA pretreated group and more than five times higher than in ^{14}C-CLA pretreated group (Fig. 4).

ODC activity. In order to determine the ability of CLA to support TPA-induced cell proliferation, ODC activity was measured in keratinocytes pretreated with fatty acid then treated with TPA. TPA-induction of ODC activity was significantly higher than acetone ($P < 0.05$) in all fatty acid-treated groups (Fig. 5). However TPA-induced ODC activity was sig-

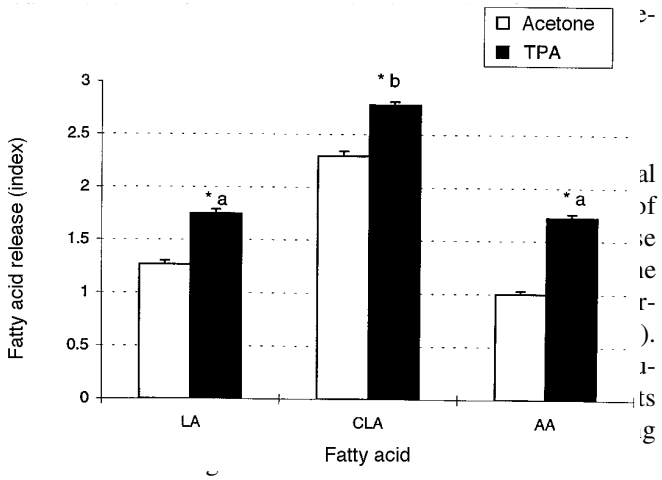

FIG. 3. 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated fatty acid release. Cultures were labeled with 0.2 μCi/mL ^{14}C-LA, ^{14}C-CLA, or ^{14}C-AA. After 12 h, cultures were changed to the media containing acetone or TPA and then incubated 3 h. Radiolabeled fatty acid release was measured in an aliquot of media and counted by liquid scintillation. An index of 1.00 was used for cultures prelabeled with ^{14}C-AA and then treated with acetone. Data represent the mean of radiolabeled fatty acid release index ± SEM from two independent experiments containing triplicate samples. Within the cultures prelabeled with same ^{14}C-fatty acid, an asterisk (*) indicates a significant difference between means of TPA treatment and acetone treatment ($P < 0.05$). Within the cultures treated with TPA, the bar not with a different letter was significantly different ($P < 0.05$). See Figure 1 for other abbreviations.

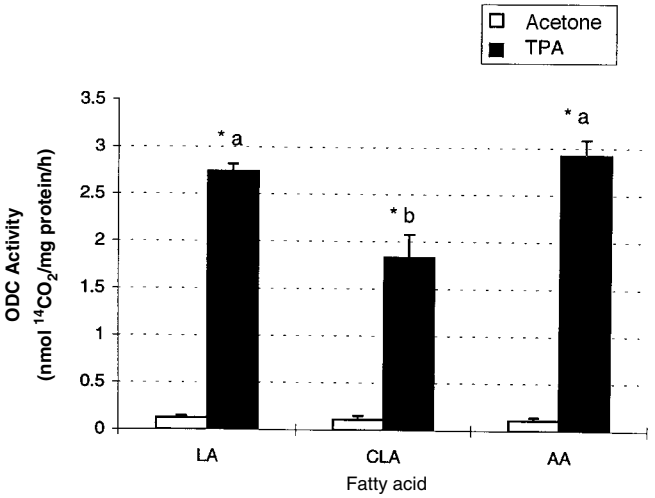

FIG. 5. Effects of LA, CLA, and AA on TPA-induced ornithine decarboxylase (ODC) activity. Confluent keratinocyte cultures were grown in serum-free media containing LA, CLA, or AA for 12 h followed by treatment with acetone or TPA for 6 h. Data represent the mean of ODC activity ± SEM from two independent experiments containing four samples. Within the cultures grown with fatty acid, an asterisk (*) indicates a significant difference between means of TPA treatment and acetone treatment ($P < 0.05$). Within the TPA-treated cultures, the bar not sharing the same letter indicates a significant difference ($P < 0.05$). See Figures 1 and 3 for other abbreviations.

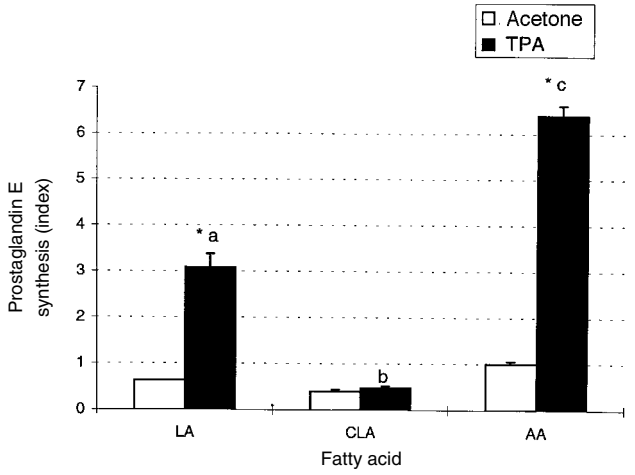

FIG. 4. TPA-stimulated prostaglandin E (PGE) synthesis. Cultures were labeled with 0.2 μCi/mL ^{14}C-LA, ^{14}C-CLA, or ^{14}C-AA. After 12 h, cultures were changed to the media containing acetone or TPA and then incubated 3 h. Radiolabeled prostaglandins were extracted from media and PGE was separated by TLC and counted by liquid scintillation. An index of 1.00 was used for cultures prelabeled with ^{14}C-AA and then treated with acetone. Data represent the mean of radiolabeled PGE synthesis index ± SEM from two independent experiments containing triplicate samples. Within the cultures prelabeled with same ^{14}C-fatty acid, an asterisk (*) indicates a significant difference between means of TPA treatment and acetone treatment ($P < 0.05$). Within the cultures treated with TPA, the bar not sharing the same letter was significantly different ($P < 0.05$). See Figures 1, 2, and 3 for abbreviations.

Exogenous CLA was incorporated into cellular lipids in a manner similar to LA and AA. However, of the phospholipid-associated fatty acids, LA and CLA were preferentially incorporated into PC while the AA was more evenly distributed into PC, PS/PI, and PE. These data are similar to previous findings showing LA and AA distribution in primary epidermal cells (13). Because it is thought that the TPA-induced cytosolic phospholipase A_2 shows preference for PC compared with other phospholipids (18,19), the predominance of LA and CLA in PC is important. However, more recent evidence suggests that this form of cytosolic phospholipase A_2 may also prefer AA-containing PC over other PC containing other fatty acids at the *sn*-2 position (i.e., LA or CLA) (19). These data are notable since we found that TPA treatment was associated with elevated levels of CLA in the media compared with LA and AA. However, there was also a higher amount of free CLA in the acetone (control) media. These data may be reflective of slightly lower incorporation of ^{14}C-CLA and/or higher turnover of CLA-containing phospholipids. The significance of elevated CLA in media of both TPA- and non-TPA (acetone)-treated cultures deserves further attention.

A large body of evidence suggests that eicosanoids play an important role in carcinogenesis and particularly in TPA-induced ODC activity and skin tumor promotion (20). In particular, AA-derived eicosanoids enhance tumor promotion-associated events in mouse skin. Previous studies demonstrated that the inhibition of TPA-induced prostaglandin synthesis reduced ODC activity which may be completely reversed by topical treatment with AA-derived PGE_2 prior to

TPA application (11). Furthermore, TPA-induced PGE_2 synthesis and ODC activity in cultures pretreated with AA were higher compared with LA (13). The tumor promoter, TPA, may stimulate epidermal cells to synthesize PGE by increasing phospholipase A_2 activity and/or inducing PGE synthase activity (20). While the CLA release induced by TPA treatment was elevated, TPA-induced PGE synthesis was low. These data support the hypothesis that dietary CLA inhibits tumor promoter-related events in keratinocytes *via* a PGE-mediated mechanism.

The action of free CLA in cells is still unknown. However, we have shown that CLA may act as a substrate of Δ6 desaturase in a manner similar to LA (21). These data may explain our findings showing a slight amount of an eicosanoid product that comigrated with PGE in our TLC solvent system. The significance of elongated/desaturated products and/or the interaction of CLA as an inhibitor of eicosanoid enzymes such as prostaglandin synthases is not presently understood.

In multistage mouse skin carcinogenesis, TPA-induced promotion is associated with increased ODC activity (9). Increasing basal levels of ODC activity result in the synthesis of polyamines which are thought to be required for stabilizing increased DNA synthesis involved with hyperproliferative cells and tumor promotion (22–24). That CLA supported lower levels of TPA-induced ODC activity than AA or LA suggests CLA is less able to support hyperproliferation induced by TPA.

These data suggest that the antipromoter activity of CLA is dependent, in part, on inhibition of TPA-induced PGE synthesis. Because dietary lipids are a complex mixture of fatty acids, further studies are needed to determine the extent that CLA competes with nonconjugated lipids that are known to modulate carcinogenesis, such LA and AA.

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Conversion of 18:3 Δ9*cis*, 12*cis*, 15*trans* in Rat Liver Microsomes

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ABSTRACT: Several years ago, it was established that the Δ15 *trans* isomer of α-linolenic acid is converted *in vivo* into fatty acids containing 20 and 22 carbons (geometrical isomers of eicosapentaenoic and docosahexaenoic acids). The present study focused on the *in vitro* Δ6 desaturation, the first step of the biosynthesis of the n-3 long-chain polyunsaturated fatty acids from 18:3n-3. For that purpose, rat liver microsomes were prepared and incubated with radiolabeled 18:3 Δ9*cis*,12*cis*,15*cis* (18:3*c,c,c*) or 18:3 Δ9*cis*,12*cis*,15*trans* (18:3*c,c,t*) under desaturation conditions. The data show that 18:3*c,c,t* is converted at a lower rate compared with α-linolenic acid. The product of conversion of 18:3*c,c,t* may be 18:4 Δ6*cis*, 9*cis*, 12*cis*, 15*trans* resulting from a Δ6 desaturation of the *trans* substrate. Moreover, the conversion of radiolabeled 18:3*c,c,t* was strongly decreased by the presence of 18:3*c,c,c* (up to 48%) while the 18:3*c,c,t* only slightly decreased the conversion of radiolabeled 18:3*c,c,c*. Thus, the desaturation enzyme presented a higher affinity for the native all-*cis* n-3 substrate.
Lipids 32, 731–735 (1997).

Heat treatments of vegetable oils induce the formation of geometrical isomers of essential polyunsaturated fatty acids (PUFA) (1,2). Linoleic acid (18:2 Δ9*cis*, 12*cis*) is mainly isomerized into 18:2 Δ9*cis*, 12*trans* and 18:2 Δ9*trans*, 12*cis* while α-linolenic acid (18:3 Δ9*cis*, 12*cis*, 15*cis*, 18:3*c,c,c*) is transformed mainly into 18:3 Δ9*trans*, 12*cis*, 15*cis* (18:3*t,c,c*) and 18:3 Δ9*cis*, 12*cis*, 15 *trans* (18:3*c,c,t*). Di-*trans* isomers also are obtained at high temperatures (above 200°C), and very little isomerization takes place at the Δ12 position of α-linolenic acid (1,2). Some years ago, it was demonstrated that the 18:3*c,c,t* isomer is the metabolic precursor of geometrical isomers of eicosapentaenoic and docosahexaenoic acids, with a *trans* double bond at the Δ17 and Δ19 positions, respectively (3). This suggests that 18:3*c,c,t* can be desaturated and elongated in rat tissues. The resulting long-chain n-3 *trans* isomers are incorporated in all tissues from rats (4) and have been recently reported in human platelets (5). Moreover, the *trans* C_{20} and C_{22} PUFA have specific physiological effects, as suggested by studies on human platelet aggregation (6,7) or rat electroretinography (4). On the other hand, it has been well established that the transformation of C_{18} PUFA into C_{20} and C_{22} long-chain metabolites is regulated at the first step of the desaturation–elongation process which is the Δ6 desaturation (8). Furthermore, a previous study has shown that a diet rich in *trans* isomers of α-linolenic acid (heated linseed oil) alters the biosynthesis of arachidonic acid at the Δ5 desaturation step (9). This suggests that an interference between the n-6 and *trans* n-3 fatty acid metabolism may exist. Alteration of the Δ6 desaturation of α-linolenic acid itself also was observed when the animals received a diet containing a purified *trans* isomer fraction (10).

However, the conversion of the 18:3*c,c,t* isomer had not yet been studied. The present investigation reports the *in vitro* conversion of 18:3*c,c,t* in microsomal fractions from livers of rats fed a fat-free diet.

MATERIALS AND METHODS

Fatty acids. The 18:3*c,c,c* fatty acids were purchased from Sigma Chemical Company (L'Isle d'Abeau, France) and NEN (Les Ulis, France) in unlabeled and [1-^{14}C]-radiolabeled (specific activity = 52.5 mCi · mmol^{-1}) forms, respectively. Unlabeled and labeled (specific activity = 51.5 mCi · mmol^{-1}) 18:3*c,c,t* was obtained by total synthesis as previously reported (11). Each radiolabeled 18:3 fatty acid was diluted in ethanol with the corresponding unlabeled compound in order to obtain a specific activity of 10 mCi · mmole^{-1}. Coenzymes and other biochemicals were purchased from Sigma (St. Louis, MO).

To identify the product of desaturation of 18:3*c,c,t*, a mixture of mono-*trans* isomers of 18:4n-3 fatty acid was prepared as follows. About 50 g of black currant seed oil (SIO, Saint Laurent Blangy, France) was saponified and the fatty acids were transformed into methyl esters, using sulfuric acid in methanol (0.45% by vol). The fatty acid methyl esters (FAME) (including about 3% of 18:4) were submitted to a urea adduction (1:3, w/w). After extraction by hexane, the

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Abbreviations: CPS, counts per second; FAME, fatty acid methyl esters; FID, flame-ionization detector; GC, gas chromatography; HPLC, high-performance liquid chromatography; I/S, inhibitor/substrate; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography.

FAME were fractionated by reversed-phase high-performance liquid chromatography (HPLC). Briefly, a Nucleosil C18 (25 cm × 10 mm i.d.) column (Shandon, Eragny, France) and a Waters R410 refractometer (Milford, MA) were used. The mobile phase was a mixture of acetonitrile and acetone (9:1, vol/vol) at a flow rate of 4 mL · min^{-1}. The purified 18:4n-3 FAME was isomerized using nitrous acid (12). Briefly, 20 mg of 18:4 FAME were heated at 40°C for 5 min in the presence of 200 µL of sodium nitrite and 130 µL of nitric acid. After the addition of water, the FAME were extracted with hexane. The *trans* FAME were then fractionated using silver nitrate thin-layer chromatography (TLC) (2). After elution with a mixture of diethyl ether and methanol (9:1, vol/vol), the plates were sprayed with a solution of 2′,7′–dichlorofluorescein in ethanol (2% by vol) and viewed under ultraviolet light. The spot corresponding to the mono-*trans* isomers (R_f = 0.31) was scraped off and extracted from the silica gel according to the method of Hill *et al.* (13), slightly modified (3). This fraction containing the mixture of the four mono-*trans* isomers was added to the samples before HPLC separation in order to collect the appropriate fraction before counting.

Preparation of microsomes and incubations. Wistar weaning male rats (IFFA CREDO, L'Arbresle, France) were fed a fat-free diet for 3 wk. After an overnight fasting period, they were sacrified by cervical dislocation. Livers were quickly excised and liver microsomes were prepared as previously described (14). Briefly, about 3.5 g of each liver was homogenized in sucrose and phosphate buffer (pH 7.4). The homogenate was then centrifuged for 20 min at 13,000 × *g*. The supernatant was centrifuged for 60 min at 100,000 × *g*. The pellet was then resuspended into saccharose–phosphate buffer and cytosol. Microsomal proteins were quantified according to Layne (15).

Microsomal suspensions containing 5 mg protein were immediately incubated with different levels (range 20–120 nmoles) of [1-^{14}C]-18:3*c,c,c* or -18:3*c,c,t*. The incubations were carried out at 37°C for 15 min, as previously described (10), with the following modifications. Each incubation medium contained 72 mM phosphate buffer, 4.8 mM $MgCl_2$, 0.5 mM CoA, 3.8 mM ATP, 0.5 mM NADPH, 0.8 mM NADH, and 0.5 mM reduced glutathione, in a final volume of 2 mL. The reciprocal interaction between both 18:3 fatty acids was also studied, using a radiolabeled substrate (60 nmoles) and its corresponding unlabeled isomer (20–60 nmoles).

Analysis of the products of conversion. The reactions were stopped by addition of a mixture of chloroform and methanol (2:1, vol/vol). After evaporation to dryness of the chloroform phase, the fatty acids were transesterified according to Morrison and Smith (16). The FAME were then extracted with hexane and fractionated using reversed-phase HPLC according to Narce *et al.* (17). Briefly, a Hibar Lichrocart superspher RP-18 (25 cm × 4 mm i.d.) column (Merck, Darmstadt, Germany) and a Waters R401 refractometer were used. The mobile phase was a mixture of acetonitrile and water (95:5, vol/vol) at a flow rate of 1 mL · min^{-1}. The unlabeled mixture of 18:3*c,c,c* and 18:3n-3 or 18:3*c,c,t* and 18:4 mono-*trans* isomers was added to each sample in order to collect the radioactive fractions which contain the remaining substrate (18:3 *cis* or *trans*) and the subsequent metabolite (18:4 *cis* or *trans*). Each collected fraction was then transferred into a scintillation vial, and Ultima Gold (Packard, Groningen, The Netherlands) scintillation cocktail was added. The radioactivity was assessed using a 1900 TR Tricarb liquid scintillator (Packard). The distribution between substrate and product (percentage of conversion) allows the calculation of the amount of substrate transformed.

*Identification of the metabolite of the conversion of Δ18:3 9*cis*, 12*cis*, 15*trans*.* In order to identify the desaturation product of 18:3*c,c,t*, incubations were performed using 0.83 µCi of radiolabeled 18:3*c,c,t* or 18:3*c,c,c* for each incubation (total: 50 µCi). After 30 min of incubation, the reaction was stopped, and the FAME were prepared as described above and pooled. They were then fractionated by HPLC as described above and then analyzed by TLC. The FAME were separated on silica gel plates (Merck) impregnated with a solution of silver nitrate in acetonitrile. After elution with diethyl ether, the plates were scanned using a Berthold (Elancourt, France) LB 2852 scanner in order to detect the radioactive products. Unlabeled standard FAME (18:3*c,c,t* and 18:4 mono-*trans* isomers) were detected under ultraviolet light after spraying the same plates with a solution of 2′,7′ dichlorofluorescein.

The FAME were also analyzed on a Hewlett-Packard 5890 series II gas chromatograph (Palo Alto, CA), equipped with a split injector and a fused Stabilwax wide-bore silica column (60 m × 0.53 mm i.d., film thickness: 0.50 µm; Restek, Evry, France). The column was connected to a flame-ionization detector (FID) and to a radio-gas chromatography-(GC)-detector (GC ram; Lablogic, Sheffield, United Kingdom). The output flow from the column was split between the FID and the radio-GC-detector. Of the effluent from the column, 90% was oxidized through copper oxide to transform labeled fatty acids into $^{14}CO_2$. The radioactivity was determined by counting $^{14}CO_2$ after mixing it with a mixture of argon and methane (9:1). The remaining 10% of effluent was used for the FID. The data were computed using the Laura software (Lablogic, Sheffield, United Kingdom).

RESULTS AND DISCUSSION

In the present work, we have studied the conversion of one of the main *trans* isomers of α-linolenic acid, which is formed during heat processing of oils. We have also compared its conversion to that of 18:3*c,c,c*, using the enzymatic desaturation system present in rat liver microsomes. In these experimental conditions, the metabolite of 18:3 is 18:4, whereas the latter do not accumulate in an *in vivo* situation.

The effects of the substrate level on the conversion by the microsomal preparation are presented in Table 1. The Δ6 desaturation rate of 18:3*c,c,c* increased rapidly up to the 60 nmoles of substrate level (3.30 nmoles of conversion product). That is the saturating substrate level, as shown by the plateau observed between 60 and 120 nmoles of substrate concentration. On the other hand, the conversion of the 18:3*c,c,t* isomer is lower than that of its *cis* homolog (1.8 vs. 3.3 nmoles with

TABLE 1
Conversion (%) and Conversion Product (nmoles) of Radiolabeled 18:3*c,c,c* and 18:3*c,c,t* by Rat Liver Microsomes[a]

^{14}C Substrate (nmoles)	18:3*c,c,c*		18:3*c,c,t*	
	Percentage conversion	nmoles	Percentage conversion	nmoles
20	12.4 ± 1.5	2.48	5.9 ± 1.7	1.18
40	7.4 ± 0.8	2.95	4.0 ± 1.1	1.60
60	5.5 ± 0.6	3.30	3.0 ± 0.4	1.80
120	2.8 ± 0.4	3.36	1.8 ± 0.3	2.16

[a]The conditions of incubation were those described in the Materials and Methods section. Results are expressed as means ± SD of four determinations; *c, cis*; *t, trans*.

60 nmoles substrate), and it did not reach a plateau. This suggests that the saturating substrate concentration may be higher for the *trans* vs. the *cis* 18:3 fatty acid in our experimental conditions.

In similar conditions, it has been demonstrated that the 18:3*c,c,c* (α-linolenic acid) is desaturated at the Δ6 position into 18:4 Δ6*cis*, 9*cis*, 12*cis*, 15*cis* (stearidonic acid). However, the metabolic product of the 18:3*c,c,t* by microsomal incubations under the present experimental conditions still remained to be totally identified. Unfortunately, the bioconver-

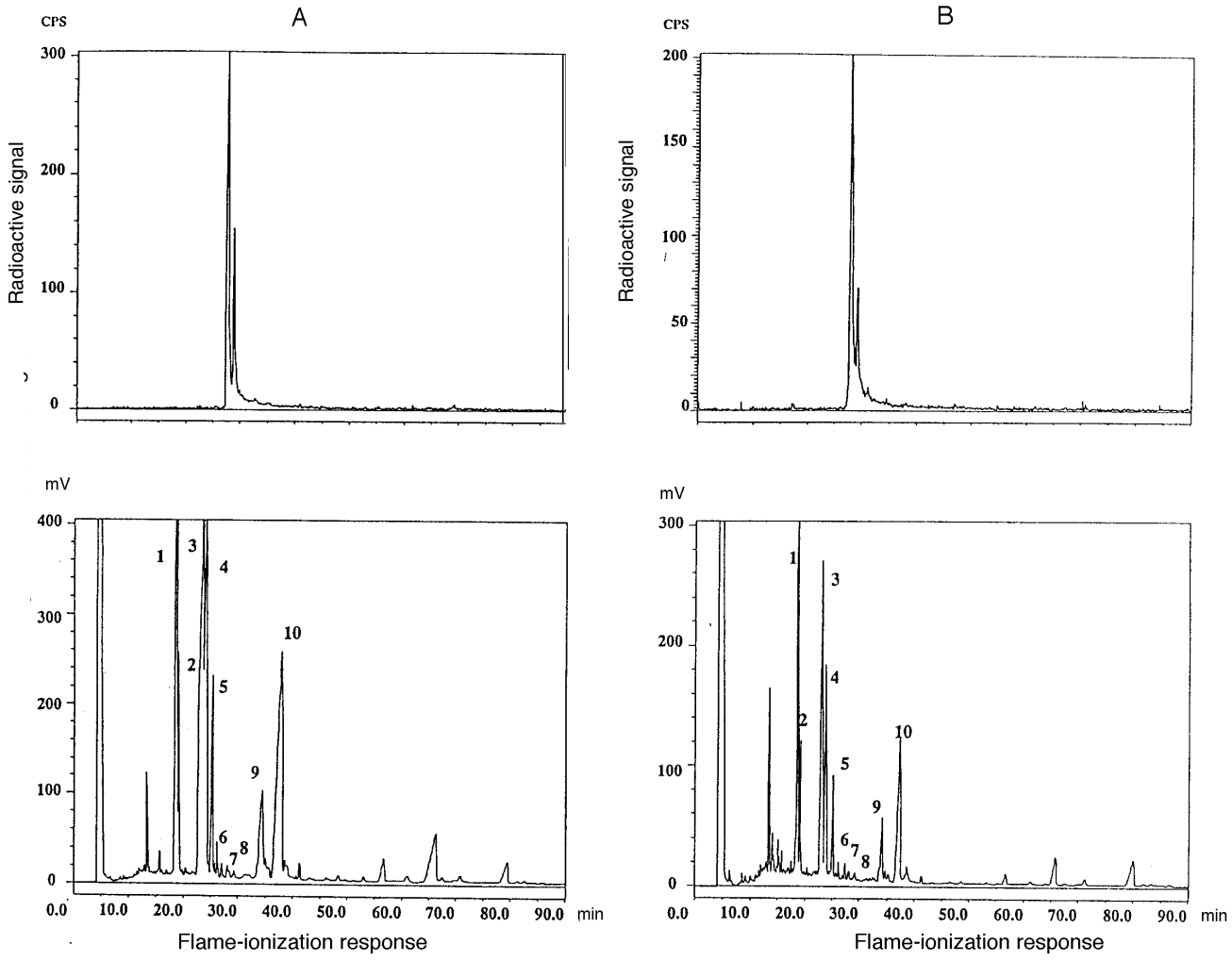

FIG. 1. Gas–liquid chromatographic analysis of the fatty acid methyl esters (FAME) from liver microsomes (5 mg protein) incubated for 15 min with [1-^{14}C]-18:3: A: FAME from liver microsomes incubated in the presence of 18:3*c,c,c*. B: FAME from liver microsomes incubated in the presence of 18:3*c,c,t*. The FAME identified are the following: 1: 16:0; 2: 16:1; 3: 18:0; 4: 18:1; 5: 18:2n-6; 6: 18:3n-6; 7: 18:3n-3; 8: 18:4n-3; 9: 20:3n-9; 10: 20:4n-6; CPS, counts per second.

TABLE 2
Reciprocal Interaction in the Conversion of 60 nmoles of Labeled 18:3 Substrates (S) in the Presence of Increasing Levels of Their Unlabeled Isomer Considered as Inhibitor (I) by Rat Liver Microsomes (nmoles of conversion product and percentage of conversion)[a]

	[^{14}C] 18:3*c,c,c*		[^{14}C] 18:3*c,c,t*	
Substrate I/S	nmoles	Percentage conversion	nmoles	Percentage conversion
1:3	3.08 ± 0.16	5.1	1.14 ± 0.18	1.9
2:3	2.89 ± 0.10	4.8	0.93 ± 0.08	1.6
1	2.58 ± 0.12	4.3	0.94 ± 0.06	1.6

[a]The conditions of incubation were those described in the Materials and Methods section. Results are expressed as average ± the range of two separate microsomal preparations., The quantities of [^{14}C] substrate transformed in the absence of inhibitor were indicated in Table 1. See Table 1 for other abbreviations.

sion of this *trans* PUFA is low, and the quantity of the metabolite was not sufficient to purify and identify this fatty acid using classical chemical methods (18). As a consequence, we have identified this product by the procedures described in the Materials and Methods section. First, the radiolabeled 18:3*c,c,t* and its metabolite were separated by $AgNO_3$–TLC (R_f = 0.79 and R_f = 0.69, respectively). Unlabeled 18:3*c,c,t* and the mixture of 18:4 mono-*trans* isomers were also eluted as standards on the same plates. The mixture of isolated mono-*trans* isomers of 18:4n-3 presented the same R_f (0.68) as the unknown metabolite. This suggests that (i) it was not an elongation product as a molecule containing three double bonds would have had a similar R_f as the 18:3*c,c,t* precursor and (ii) it is a molecule containing four ethylenic bonds, one of them being in *trans* configuration. Additionally, the radio-GC-detection allowed us to see two labeled compounds (Fig. 1) for the FAME from liver microsomes incubated either in the presence of [1-^{14}C]-18:3 Δ9*cis*, 12*cis*, 15*trans* or in the presence of [1-^{14}C]-linolenic acid. The second peak was not detected when 18:3*c,c,t* was analyzed under similar conditions (data not shown). These two peaks presented the same retention time as 18:3 and 18:4, respectively. Together, these data (HPLC, TLC, GC) clearly indicate that the Δ15 *trans* isomer of linolenic acid is desaturated into a 18:4 *trans* fatty acid. Unfortunately, the amount of the metabolite formed was not sufficient to identify the position of the new double bond by techniques such as GC–mass spectrometry analysis of the dimethyl oxazoline derivatives (19). However, previous data suggested that it cannot be a Δ5 double bond as the metabolite of 18:3*c,c,t*, the 20:5 Δ5*cis*, 8*cis*, 11*cis*, 14*cis*, 17*trans* requires a Δ6 desaturation of 18:3*c,c,t* before an elongation and Δ5 desaturation (3).

The reciprocal interaction in the *in vitro* conversion of both 18:3 fatty acids is presented in Table 2. The 18:3*c,c,c* decreased the conversion of 18:3*c,c,t*. This decrease was more than 36% at an inhibitor/substrate (I/S) ratio of 1:3, reaching a maximum value of 48% at an I/S ratio of 2:3. On the other hand, the *trans* isomer slightly decreased the Δ6 desaturation of 18:3*c,c,c*. This effect ranged from 7 to 22% as the I/S ratio increased from 1:3 to 1. There was therefore a preferential desaturation of the 18:3*c,c,c*. These results suggest that the affinity of the enzyme is greater for the all-*cis* molecule as compared to the *trans* one.

These *in vitro* data have to be confirmed in cellular structures using hepatocytes in culture and *in vivo* experiments. These experiments will allow a better understanding of the metabolic pathway of such unusual fatty acids.

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Increased Hepatic β-Oxidation of Docosahexaenoic Acid, Elongation of Eicosapentaenoic Acid, and Acylation of Lysophosphatidate in Rats Fed a Docosahexaenoic Acid-Enriched Diet

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ABSTRACT: Rats were fed a diet supplemented with corn oil (n-3 deficient), soy oil, or a mixture containing 8% 22:6n-3 ethyl ester for 6 wk. The hepatic capacities for the β-oxidation and synthesis of 22:6n-3, in addition to the acylation of lysophosphatidate, were tested *in vitro*. In rats that were fed a 22:6n-3-enriched diet, both the β-oxidation of 22:6n-3 and elongation of 20:5n-3 were enhanced compared to those in rats fed the other diets. Acylation of lysophosphatidate was also enhanced in rats fed a 22:6n-3-enriched diet, while the rate of dephosphorylation of phosphatidate was not changed. The amount of 22:6n-3 in the liver was much less than that consumed in a docosahexaenoic acid-enriched diet. These results suggest that a significant amount of dietary 22:6n-3 was degraded *via* β-oxidation, and that a portion of the retroconverted 20:5n-3 was recycled for the synthesis of 22:6n-3. The recycling of 20:5n-3 might contribute to the low level of 22:6n-3 in rats fed an n-3-deficient diet.

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Docosahexaenoic acid (DHA, 22:6n-3) in biomembranes is known to be essential for maintaining visual function in monkeys (1,2) and for improving the learning ability of rats (3,4). A prolonged depletion of n-3 fatty acid has been associated with neurological disturbance in a 6-yr-old girl (5). Maintaining the level of 22:6n-3 in biomembranes seems to be important for normal neurological function. It has been suggested that dietary 22:6n-3 is incorporated intact into brain phospholipid (PL) (6), while 18:3n-3 is converted to 22:6n-3 in the liver and then incorporated into brain PL (7). We previously reported that the fatty acid composition of cerebral cortical gray matter was slightly affected by the continuous intake of 22:6n-3 in rats after weaning, although this dietary effect was more distinct in the suckling period (8). We suggested that dietary 22:6n-3 mainly accumulated in the liver after weaning when liver growth accelerated. Furthermore, Anderson and Connor (9) reported that most of the radioactivity of labeled 22:6n-3 was found in the liver of the suckling rat 30 min after injection into the jugular vein, although the radioactivity in the liver was considerably lower in rats that had been functionally hepatectomized. With regard to the metabolism of 22:6n-3, the rates of both its synthesis and degradation seem to be slow and respond to the intake of only a small amount of n-3 fatty acid, considering that 22:5n-3, but not 22:6n-3, accumulates in various cells when they are incubated with 18:3n-3 (10–12), and that a low level of 22:6n-3 is maintained in the rat liver and brain after treatment with an n-3-deficient diet for several generations (13,14). The continuous intake of 22:6n-3 influences the rate of 22:6n-3 metabolism in organs, especially in the liver, which is an important organ for lipid metabolism and storage. The administration of 22:6n-3 (0.3 or 1.0 mL/d) to rats for 14 d induces an increase in β-oxidation and reduces Δ-5 and Δ-6 desaturations of n-6 fatty acid in hepatocytes but does not affect the formation of 22:6n-3 from 20:5n-3 (15). In addition, hepatic peroxisomal β-oxidation is enhanced by the administration of a large amount of 22:6n-3 (500–1500 mg/d) for 10 d in rats, while mitochondrial β-oxidation is not (16). In the present study, the effect of the continuous intake of 22:6n-3 on its own metabolism was tested in rats that were fed a lower level of 22:6n-3 (120–150 mg/d) for a longer period (6 wk) than in previous studies (15,16). Assays were performed when the fatty acid compositions reached a steady state in organs of rats fed different fats. The replacement of fatty acids requires at least 2 wk in livers and longer in the other organs of rats after their diet is changed (14). β-Oxidation of 22:6n-3, synthesis of 22:6n-3, and acylation of lysophosphatidate (LPA) with 22:6n-3 were tested *in vitro* to evaluate the effectiveness of the continuous intake of 22:6n-3 in maintaining 22:6n-3 levels in organs.

MATERIALS AND METHODS

Animals. Male 4-wk-old Sprague-Dawley Rats (Funabashi Farm, Funabashi, Chiba, Japan) were fed 20–25 g/d of a semipurified diet containing 7% fat (17). Table 1 shows the fatty

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Abbreviations: BSA, bovine serum albumin; DHA, docosahexaenoic acid; HPLC, high-performance liquid chromatography; LCPUFA, long-chain polyunsaturated fatty acid; LPA, lysophosphatidate; PA, phosphatidate; PAP, phosphatidate phosphohydrolase; PL, phospholipid; TBARS, thiobarbituric acid reactive substance; TG, triglyceride; TLC, thin-layer chromatography.

acid compositions of the dietary fats. After the 6-wk feeding period, the rats were fasted for 15 h and decapitated under diethyl ether anesthesia. Blood was collected in a vial containing heparin, and livers were removed after perfusion with saline, frozen in liquid N_2, and stored at –80°C until use. The procedure used in this study complied with the guide of our institute for the care and use of laboratory animals.

Materials. Radiolabeled fatty acids (1.90–2.15 GBq/mmol) were obtained from New England Nuclear (Boston, MA) and diluted with unlabeled fatty acids (Funakoshi, Tokyo, Japan). 1-Palmitoylglycero-3-phosphate and phosphatidate (PA) were obtained from Funakoshi. Defatted bovine serum albumin (BSA) and malonyl-CoA were obtained from Sigma (St. Louis, MO). Other chemicals were purchased from Wako Pure Chemical (Osaka, Japan).

Ketone body determination. Plasma acetoacetate and 3-hydroxybutyrate levels were measured colorimetrically (18) using a commercial kit (Sanwa Chemical, Nagoya, Aichi, Japan).

Malonyl-CoA determination. The assay was performed basically according to the procedure described by Demoz *et al*. (19). The liver was homogenized in 5% sulfosalicylic acid containing 50 mM dithiothreitol, and centrifuged at 700 × *g* for 10 min. An aliquot of the supernatant was filtered through a 45-µm filter and applied to a high-performance liquid chromatography (HPLC) system (Japan Spectroscopic, Tokyo, Japan) fitted with a LiChrosorb RP-18 column (5-µm particle size, 4 × 250 mm; Merck, Darmstadt, Germany). The solvent contained 6% (vol/vol) methanol in 100 mM sodium dihydrogen phosphate and 75 mM sodium acetate adjusted to pH 4.7, and was delivered at a rate of 1.5 mL/min. Peaks were monitored at 254 nm with a spectrophotometric detector, and the concentration of malonyl-CoA was calculated from the peak area by comparison to a standard curve.

Preparation of homogenates. The liver was homogenized in 0.25 M sucrose and 2 mM $MgCl_2$/3 mM Tris-HCl buffer solution (pH 7.4) and centrifuged at 700 × *g* for 5 min. The supernatant was used for the enzyme assays. The protein content of the supernatant was measured using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). To assess subcellular densities in the homogenate solution, the activities of catalase (20), cytochrome c oxidase (21), and NADPH cytochrome c reductase (22) were measured as markers for peroxisomes, mitochondria, and microsomes, respectively.

β-Oxidation rate. The oxidation of fatty acids to acetate was measured by a modification of the procedure described by Singh *et al*. (23). A final volume of 250 µL of 50 mM Tris-HCl buffer solution (pH 8.0) contained 37.5 µmol KCl, 0.5 µmol $MgCl_2$, 0.5 µmol oxaloacetate, 1.25 µmol ATP, 0.125 µmol dithiothreitol, 50 nmol NAD, 50 nmol L-carnitine, 15 nmol FAD, 15 nmol $CoANa_3$,10 nmol [1-^{14}C]18:1n-9 or [1-^{14}C]18:2 (70 Bq/nmol), and 0.5 mg BSA. The reaction was started by adding homogenate solution (135–155 µg protein) and stopped after incubation at 37°C for 3 min by adding 0.5 mL of 1 M KOH. The reaction mixture was heated at 60°C for 1 h to hydrolyze acyl-CoA and acyl-carnitine, and acidified with 50 µL of concentrated HCl. After removing fatty acid by the extraction procedure of Folch *et al*. (24), the aqueous phase was transferred to a scintillation vial containing scintillation fluid, and counted in a liquid scintillation analyzer (model 1900 TR; Packard Instrument, Meriden, CT). When [1-^{14}C]20:5n-3 or [1-^{14}C]22:6n-3 (120 or 100 Bq/nmol) was used as a substrate, 25 nmol NADPH was added to the reaction mixture without oxaloacetate. A blank assay was performed using boiled homogenates.

DHA synthesis. The synthesis of 22:6n-3 from 20:5n-3 was measured as previously reported, with minor modifications (25). A final volume of 500 µL of 50 mM potassium phosphate buffer solution (pH 7.4) contained 75 µmol KCl, 2.5 µmol ATP, 2.5 µmol $MgCl_2$, 0.75 µmol glutathione reduced form, 0.625 µmol NADH, 0.625 µmol NADPH, 0.15 µmol $CoANa_3$, 0.15 µmol malonyl-CoA, 50 nmol [1-^{14}C]20:5n-3 (35 Bq/nmol), 0.5 mg BSA, and 0.5 mg Triton WR1339. The reaction was carried out at 37°C for 10 min after adding homogenate solution (1.2–1.4 mg protein) and stopped by adding 1 mL of 10% (wt/vol) KOH/ethanol. Butylated hydroxytoluene was added as an antioxidant and [1-^{14}C]18:0 (250 Bq/tube) was added as an internal standard, and the mixture was heated at 70°C for 60 min. After removing unsaponifiable lipids using petroleum ether, 1 mL of 6 M HCl and 1 mL of KCl-saturated solution were added to the mixture, and the fatty acids were extracted from the acidified mixture with petroleum ether. The fatty acids were analyzed with an HPLC system (Gilson Medical Electronics, Middleton, WI) fitted with two LiChrosorb RP-18 columns (5-µm particle size, 4 × 250 mm; Merck) in series. The fatty acids were eluted with 10% (vol/vol) aqueous methanol containing 0.1% (vol/vol) phosphoric acid at 1 mL/min, and monitored at 210 nm with a spectrophotometric detector, coupled in series to a Ramona radioactivity detector. Peaks were identified by comparing the retention times to those of fatty acid standards (Nippon Suisan Kaisha, Tokyo, Japan). A blank assay was performed using boiled homogenates.

Acyl-CoA synthetase. Activation of [1-^{14}C]18:2 (22 Bq/nmol) or [1-^{14}C]22:6n-3 (26 Bq/nmol) was assayed according to the procedure described by Reddy and Bazan (26).

Acylation of LPA. A final volume of 250 µL of 100 mM

TABLE 1
Fatty Acid Compositions of the Dietary Fats

Fatty acid	Corn	Soy	DHA[a]
	(wt%)		
16:0	11.2	10.1	8.0
18:0	2.2	3.7	2.0
18:1	31.5	24.0	27.2
18:2n-6	51.7	51.9	43.9
18:3n-3	0.2	6.9	0.9
20:5n-3	n.d.	n.d.	1.9
22:6n-3	n.d.	n.d.	8.3
Others	3.2	4.4	7.8

[a]Fatty acid ethyl esters prepared from marine fish oil (Maruha Co.,Tokyo, Japan), in which docosahexaenoic acid (DHA) ethyl ester was concentrated up to a level of 60%, were added to corn oil at a ratio of 15:85 (w/w). n.d., not detected.

potassium phosphate buffer solution (pH 7.4) contained 0.625 μmol ATP, 0.5 μmol $MgCl_2$, 0.125 μmol dithiothreitol, 50 nmol $CoANa_3$, 40 nmol 1-palmitoylglycero-3-phosphate, 5 nmol [1-^{14}C]18:1n-9, 18:2n-6 or 22:6n-3 (150–170 Bq/nmol), 0.2 mg BSA, and 25 μg Triton X-100. The reaction was performed at 37°C for 1–3 min after adding homogenate solution (135–155 μg protein) and stopped by adding 2 mL of chloroform/methanol (1:1, vol/vol) containing 0.1 M HCl. Carrier phosphatidate (PA) and butylated hydroxytoluene were added, and the lipids were extracted according to the procedure described by Kanoh and Åkesson (27) and applied to high-performance thin-layer chromatography (TLC) plates (28). The PA on a Silica Gel 60 aluminum plate (Merck) viewed under iodine vapor was cut out, transferred to a scintillation vial containing scintillation fluid, and counted in a liquid scintillation analyzer. A blank assay was performed without 1-palmitoylglycero-3-phosphate.

Phosphatidate phosphohydrolase (PAP). Dephosphorylation of PA was assayed in the presence of $MgCl_2$ according to the procedure described by Surette *et al.* (29).

Fatty acid analysis. Liver and plasma lipids were extracted by the method of Folch *et al*. (24) and developed on a Silica Gel 60 TLC plate containing a fluorescent indicator (Merck) with *n*-hexane/ethyl ether/formic acid (80:30:1, by vol). The triglyceride (TG) and PL bands on the TLC plates were scraped into tubes and heated at 70°C for 3 h in acetyl chloride/methanol (1:9, vol/vol) with 17:0 as an internal standard. Fatty acid methyl esters were extracted with *n*-hexane. Fatty acid methyl esters were analyzed by gas chromatography as previously described (30).

Thiobarbituric acid reactive substances (TBARS). TBARS were measured by the method of Ohkawa *et al*. (31).

Statistical analysis. Differences between groups were evaluated by the Mann-Whitney U-test when the *P* value in the Kruskal-Wallis test was less than 0.05.

RESULTS

There were no differences among the groups in either the body weight or the liver weight when the rats were sacrificed. The body weights (g) and liver weights (g), ± SD, were as follows: 372.8 ± 8.6 and 14.2 ± 2.3 in the corn group; 360.4 ± 15.8 and 11.9 ± 1.3 in the soy group; 368.6 ± 14.5 and 12.7 ± 0.9 in the DHA group, respectively.

In the rats fed a commercial chow, the rate of β-oxidation of [1-^{14}C]18:2n-6 by homogenate prepared from a frozen piece of liver was about 70% of that by homogenate prepared from a piece kept at 4°C overnight in 0.25 M sucrose and 2 mM $MgCl_2$/3 mM Tris-HCl buffer solution (pH7.4), as shown in Figure 1. However, depression in β-oxidation of [1-^{14}C]20:5n-3 in a frozen piece was much less than that of [1-^{14}C]18:2n-6, and the formation of acid-soluble product from [1-^{14}C]22:6n-3 in 5 min was not depressed (data not shown).

Table 2 shows parameters which reflect hepatic β-oxidation in the three dietary groups. No differences were observed among the dietary groups in the levels of plasma acetoacetate and 3-hydroxybutyrate, which are excreted from the liver as final products of mitochondrial β-oxidation. Liver malonyl-CoA levels were almost the same in the three groups. No differences were observed in the activities of cytochrome c oxidase and catalase, which indirectly reflect mitochondrial and peroxisomal densities in liver homogenates, respectively. Although no differences were observed among the groups in the amounts of acid-soluble products from [1-^{14}C]18:1n-9 and [1-^{14}C]18:2n-6, the amount of product from [1-^{14}C]22:6n-3 was significantly higher in the DHA group than in the other two groups ($P < 0.05$). In addition, the product from [1-^{14}C]20:5n-3 was formed at a slightly higher rate in the DHA group, but the differences between the groups were not statistically significant ($P = 0.05$). When BSA in the reaction mixture was replaced with Triton X-100 at a level of 0.1%, which suppresses mitochondrial β-oxidation much more than it does peroxisomal β-oxidation (32), the formation of acid-soluble product from both [1-^{14}C]20:5n-3 and [1-^{14}C]22:6n-3 was highest in the DHA groups (data not shown).

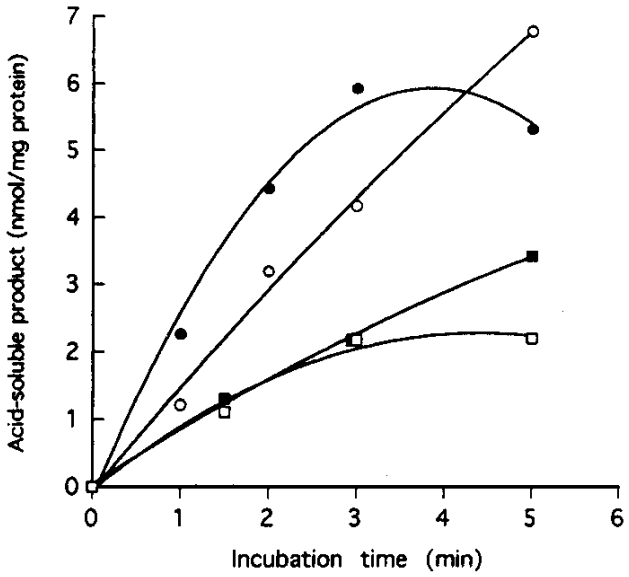

FIG. 1. The formation of acid-soluble product from [1-^{14}C]18:2n-6 and [1-^{14}C]20:5n-3 by a liver homogenate prepared from a frozen piece or a fresh piece of liver. A lobe of liver obtained from a 9-wk-old rat that had been fed a commercial diet was cut into two pieces. One piece was frozen (open symbols) and the other was kept at 4°C overnight in a buffer containing 0.25 M sucrose (closed symbols). ○,●, [1-^{14}C] 18:2n-6; □, ■, [1-^{14}C]20:5n-3.

The specific activity of NADPH cytochrome c reductase (nmol/min/mg protein) as a marker of microsomes was almost the same in the three groups: 36.6 ± 6.3 in the corn group; 36.5 ± 4.8 in the soy group; 33.0 ± 5.8 in the DHA group.

Figure 2 shows an HPLC chromatogram of 22:6n-3 synthesis from 20:5n-3 by liver homogenates of a rat fed soy oil. Two radioactive peaks, which represent elongation and desaturation products from 20:5n-3, were detected, and the radioactivity of endogenous stearic acid (18:0) was almost equal to that at baseline in the absence of [1-^{14}C]18:0 as an

TABLE 2
Hepatic β-Oxidation of Rats Fed Different Fats[a]

	Corn	Soy	DHA
Acetoacetate (μmol/mL plasma)	0.20 ± 0.05	0.16 ± 0.07	0.18 ± 0.06
3-Hydroxybutyrate (μmol/mL plasma)	0.44 ± 0.13	0.41 ± 0.09	0.31 ± 0.04
Malonyl-CoA (nmol/g liver)	29.2 ± 4.6	31.0 ± 4.8	28.7 ± 5.0
Cytochrome c oxidase activity (μmol/min/mg protein)	0.14 ± 0.03	0.15 ± 0.03	0.13 ± 0.02
Catalase activity (mmol/min/mg protein)	0.81 ± 0.11	0.74 ± 0.11	0.87 ± 0.12
Acid-soluble product from			
[1-^{14}C]18:1n-9 (nmol/min/mg protein)	1.38 ± 0.10	1.28 ± 0.05	1.37 ± 0.17
[1-^{14}C]18:2n-6 (nmol/min/mg protein)	1.24 ± 0.32	1.43 ± 0.23	1.41 ± 0.39
[1-^{14}C]20:5n-3 (nmol/min/mg protein)	0.34 ± 0.11	0.33 ± 0.09	0.46 ± 0.14
[1-^{14}C]22:6n-3 (nmol/min/mg protein)	0.47 ± 0.13[a]	0.45 ± 0.13[a]	0.65 ± 0.07[b]

[a]Values (means ± SD of five rats) with different superscript letters (a,b) are significantly different ($P < 0.05$). For abbreviation see Table 1.

internal standard (Fig. 2B). As shown in Table 3, the amounts of both 22:5n-3 and 22:6n-3 formed from 20:5n-3 were significantly higher in the DHA group than in the other two groups ($P < 0.05$). Rats in the DHA group showed a greater capacity for 22:6n-3 synthesis, mainly owing to the enhanced chain elongation of 20:5n-3. The synthesis of 22:6n-3 from 22:5n-3 seemed to be almost the same in the three dietary groups, considering that there were no differences in the ratio of 22:6n-3 to 22:5n-3 among the groups.

The effect of dietary fat on the acylation of 1-palmitoylglycero-3-phosphate is shown in Table 4. Acyl-CoA was formed from either 18:2n-6 or 22:6n-3 at a similar rate in the three groups by homogenates, as well as microsomal and mitochondrial fractions (data not shown). The rate of the incorporation of either 18:1n-9 or 18:2n-6 into the PA fraction was higher in the DHA group than in the other two groups ($P < 0.05$), and the intake of 22:6n-3 in the DHA group was more rapid than that in the soy group ($P < 0.05$). The specific activity of acylation of LPA appeared to be higher in the DHA group; however, no differences were observed in the specific activity of PAP, which is a key enzyme in TG synthesis.

The concentrations of the major fatty acids in liver TG and PL are shown in Tables 5 and 6, respectively. Long-chain polyunsaturated fatty acids (LCPUFA) were abundant in the PL fraction. In the DHA group, a considerable amount of 22:6n-3 was detected in both TG and PL, and the 22:6n-3 levels in both fractions were highest in this group. In the corn and soy groups, only a very low level of 22:6n-3 in TG was detected. The 20:4n-6 level in TG was highest in the corn group; however, no differences were observed among the groups in PL. The levels of both 20:5n-3 and 22:5n-3 in TG were highest in the DHA group. No differences were observed among the groups in the 20:5n-3 level in PL; however, the level of 22:5n-3 in PL was significantly lower in the corn group than in the other two groups. The amounts of 22:6n-3 in the plasma and in the liver were highest in the DHA group (Fig. 3). In addition, there were no differences among the

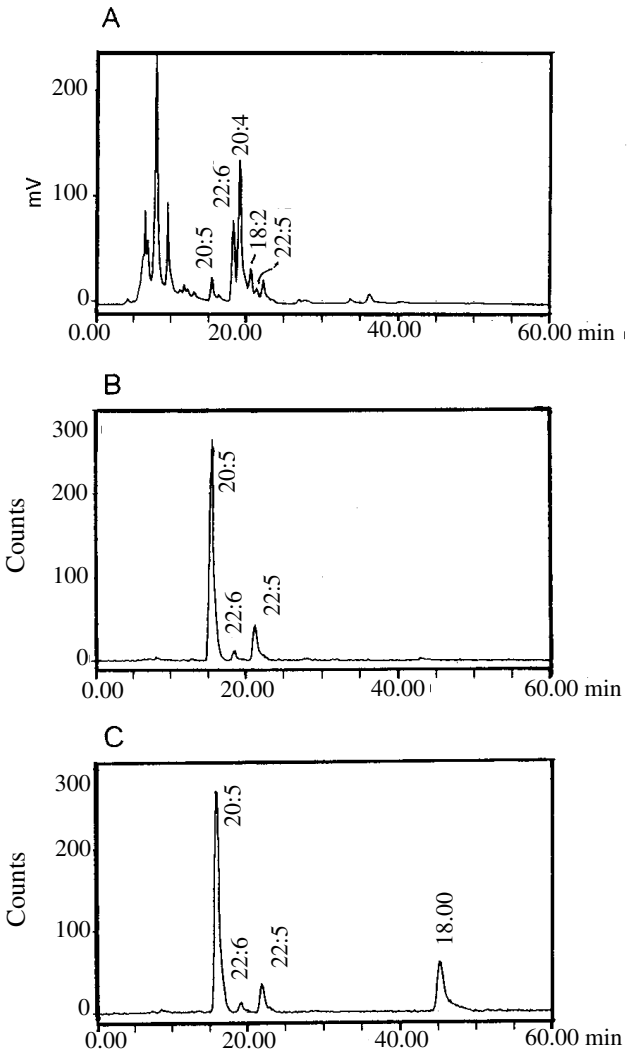

FIG. 2. High-performance liquid chromatography chromatograms of metabolites formed from [1-^{14}C]eicosapentaenoic acid by rat liver homogenates. The details of the incubation and separation procedures are described in the Materials and Methods section. A: Peaks with absorption at 210 nm; B: peaks with radioactivity (in the absence of [1-^{14}C]18:0 as an internal standard); C: peaks with radioactivity (in the presence of [1-^{14}C]18:0 as an internal standard).

TABLE 3
Production of 22:5n-3 from 20:5n-3 by Liver Homogenates of Rats Fed Different Fats[a]

	Corn	Soy	DHA
22:5n-3 (nmol/min/mg protein)	0.46 ± 0.05[a]	0.52 ± 0.06[a]	0.66 ± 0.06[b]
22:6n-3 (nmol/min/mg protein)	0.16 ± 0.03[a]	0.16 ± 0.02[a]	0.23 ± 0.05[b]
22:6n-3/22:5n-3	0.34 ± 0.05	0.30 ± 0.02	0.35 ± 0.06

[a]Values (means ± SD of five rats) with different superscript letters (a,b) are significantly different ($P < 0.05$). For abbreviation see Table 1.

TABLE 4
Activation of Fatty Acids, Acylation of 1-Palmitoylglycero-3-Phosphate and Dephosphorylation of Phosphatidate in Livers of Rats Fed Different Fats[a]

		Corn	Soy	DHA
		(nmol/min/mg protein)		
Activation of	18:2n-6	103.23 ± 22.31	106.15 ± 23.78	122.00 ± 14.82
	22:6n-3	25.60 ± 14.35	44.35 ± 15.61	35.98 ± 12.31
Acylation of LPA with	18:1n-9	9.93 ± 1.60[a]	9.06 ± 1.02[a]	12.74 ± 1.72[b]
	18:2n-6	10.01 ± 0.67[a]	9.19 ± 1.43[a]	12.02 ± 1.42[b]
	22:6n-3	1.07 ± 0.22[a,b]	0.83 ± 0.15[a]	1.38 ± 0.33[b]
Dephosphorylation of	PA	9.06 ± 1.54	6.94 ± 0.83	6.83 ± 0.68

[a]Values (means ± SD of five rats) with different superscript letters (a,b) are significantly different ($P < 0.05$). LPA, lysophosphatidate; PA, phosphatidate; for other abbreviation see Table 1.

TABLE 5
Fatty Acid Content in Liver Triglyceride of Rats Fed Different Fats[a]

	Corn	Soy	DHA
Fatty acid		(μmol/g liver)	
16:0	28.46 ± 4.83	20.39 ± 5.87	23.61 ± 2.84
18:0	1.51 ± 0.25	1.22 ± 0.31	1.73 ± 0.34
18:1	29.12 ± 4.38[a]	18.48 ± 5.99[a,b]	20.31 ± 3.19[b]
18:2n-6	14.82 ± 4.39	14.08 ± 2.47	16.24 ± 1.55
20:3n-6	0.15 ± 0.14	0.09 ± 0.08	0.18 ± 0.07
20:4n-6	2.12 ± 0.98[a]	1.32 ± 0.27[a,b]	1.04 ± 0.20[b]
20:5n-3	trace[a]	0.14 ± 0.08[b]	1.19 ± 0.29[c]
22:4n-6	0.47 ± 0.09[a]	0.27 ± 0.07[b]	0.19 ± 0.04[c]
22:5n-6	0.23 ± 0.05[a]	0.05 ± 0.02[b]	0.10 ± 0.02[c]
22:5n-3	trace[a]	0.22 ± 0.04[b]	0.98 ± 0.18[c]
22:6n-3	trace[a]	0.38 ± 0.08[b]	3.87 ± 0.87[c]

[a]Values (means ± SD of five rats) with different superscript letters (a–c) are significantly different ($P < 0.05$). For abbreviation see Table 1.

groups with respect to the TBARS value (nmol/g liver), which reflects the level of the secondary product of lipid peroxidation (33): 44.3 ± 3.7 in the corn group; 50.6 ± 4.0 in the soy group; 50.9 ± 3.5 in the DHA group.

DISCUSSION

In this study, the effects of dietary n-3 LCPUFA on fatty acid oxidation were not clear, except for that on the β-oxidation of 22:6n-3. The hepatic capacity for ketogenesis might be almost the same in the three dietary groups, as suggested by the results of tests for plasma ketone bodies, in addition to cytochrome c oxidase and catalase activities (Table 2). Furthermore, the level of malonyl-CoA, which is an inhibitor of the translocation of fatty acyl residues to mitochondria (34), was also the same in the three groups. These results suggest that a low-fat diet over a longer period than has been used in previous studies (15,16,29,35–38) does not produce a remarkable change in the capacity for hepatic ketogenesis. Dietary n-3 LCPUFA is known to increase the capacity for hepatic fatty acid oxidation; however, this effect is not clear in rats fed a diet containing less than 15% fat, and enzyme activities related to β-oxidation often depend on the duration of feeding (36).

No differences were observed among the groups in the rate of the formation of acid-soluble product from either [1-^{14}C]18:1 or 18:2. These values might reflect the initial rate of β-oxidation, i.e., the rate of the normal cycle of β-oxidation. The measurement of radioactive acid-soluble product formed from [1-^{14}C]fatty acid depends on the initial one or two cycles of β-oxidation. The rate of the formation of acid-soluble product from 22:6n-3 was significantly higher in the

TABLE 6
Fatty Acid Content in Liver Triglyceride of Rats Fed Different Fats[a]

Fatty acid	Corn	Soy	DHA
	(μmol/g liver)		
16:0	9.31 ± 0.56[a]	10.07 ± 1.02[a,b]	11.50 ± 1.19[b]
18:0	13.86 ± 0.92	14.76 ± 2.08	15.08 ± 1.22
18:1	2.94 ± 0.44	2.56 ± 0.34	2.33 ± 0.23
18:2n-6	3.61 ± 0.40[a]	4.07 ± 0.45[a]	4.71 ± 0.34[b]
20:3n-6	0.18 ± 0.03[a]	0.25 ± 0.06[a]	0.46 ± 0.09[b]
20:4n-6	14.35 ± 1.15	13.83 ± 1.72	12.80 ± 0.97
20:5n-3	0.26 ± 0.09[a]	0.22 ± 0.02[a]	0.26 ± 0.07
22:4n-6	0.48 ± 0.03[a]	0.17 ± 0.03[b]	0.07 ± 0.004[c]
22:5n-6	1.47 ± 0.11[a]	0.12 ± 0.04[b]	0.06 ± 0.01[c]
22:5n-3	0.17 ± 0.02[a]	0.44 ± 0.05[b]	0.37 ± 0.05[b]
22:6n-3	1.41 ± 0.05[a]	3.45 ± 0.56[b]	5.24 ± 0.57[c]

[a]Values (means ± SD of five rats) with different superscript letters (a–c) are significantly different ($P < 0.05$). For abbreviation see Table 1.

DHA group than in the other groups in the presence of NADPH ($P < 0.05$), while little product was formed from either 20:5n-3 or 22:6n-3 in the absence of NADPH (data not shown). The β-oxidation of fatty acids which have a Δ-4 double bond requires 2,4-dienoyl-CoA reductase (39) and proceeds beyond a double bond positioned at either an even- or an odd-numbered carbon atom as a result of the addition of NADPH or a NADPH-generating substrate (40–42). This result suggests that the activity of 2,4-dienoyl-CoA reductase was relatively increased in rats fed a DHA-enriched diet. Bergseth *et al*. (43) reported that 2,4-dienoyl-CoA reductase activity in rat hepatocytes was higher in a fish oil-fed group than in a soya oil-fed group, although there was no difference in the rate of the production of ketone bodies from 22:6n-3 between groups fed the same amounts of soy and fish oils. Willumsen *et al*. (38) reported that a single dose of 20:5n-3 ethyl ester, but not 22:6n-3, enhanced mitochondrial 2,4-dienoyl-CoA reductase. The amount of acid-soluble product from 20:5n-3 was slightly higher in the DHA group than in the other two groups ($P = 0.05$), and 20:5n-3 was found at only a low level (less than 1.5 μmol/g liver) in TG and PL combined, as shown in Tables 5 and 6. These results suggest that the β-oxidation of n-3 LCPUFA was more active in the DHA group than in the other groups and that a significant amount of 22:6n-3 was completely degraded in the DHA group.

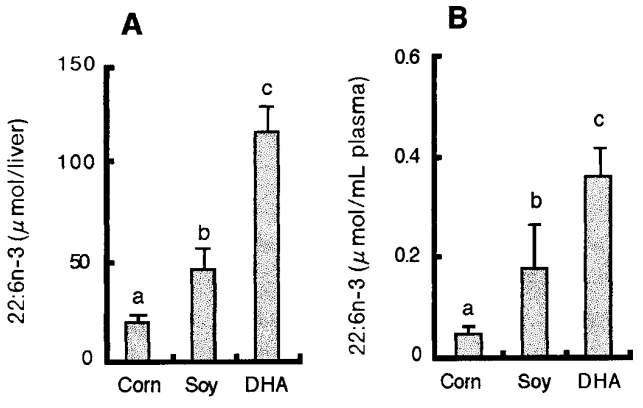

FIG. 3. The 22:6n-3 levels in liver (A) and plasma (B). Each bar represents the amount of 22:6n-3 in the combined triglyceride and phospholipid fractions (mean ± SD of five rats). Bars with different superscript letters (a–c) are significantly different ($P < 0.05$). DHA: docosahexaenoic acid.

Schlenk *et al*. (44) reported that a considerable amount of retroconverted 20:5n-3 was chain-elongated in the liver to 22:5n-3, the carboxyl group of which showed much lower radioactivity than those of 20:5n-3 and 22:6n-3, when rats were fed [U-^{14}C]22:6n-3. In rat hepatocytes, chain elongation of retroconverted 20:5n-3 is observed during prolonged incubation with [4,5-^{3}H]22:6n-3 (45). In this study, the chain elongation of 20:5n-3 *in vitro* was most active in the DHA group, and the amount of 22:6n-3 formed was also significantly higher in the DHA group than in the other dietary groups ($P < 0.05$) (Table 4). This result appears to be inconsistent with the finding of Grønn *et al*. (15), who reported that the synthesis of 22:6n-3 from 20:5n-3 in rat hepatocytes was not affected by the intake of n-3 LCPUFA. However, it should be noted that the source of a two-carbon unit for the chain elongation of 20:5n-3 in hepatocytes is limited to endogenous malonyl-CoA. The chain elongation of 20:5n-3 certainly may have been affected by the amount of substrate acid in the liver, which was most abundant in the DHA group, as shown in Tables 5 and 6. However, the 20:5n-3 levels in the livers of rats fed diets with different 18:2n-6/18:3n-3 ratios did not affect the synthesis of 22:5n-3 or 22:6n-3 from 20:5n-3 in our previous *in vitro* study with a crude microsomal fraction, which included peroxisomes (46). In rats fed a DHA-enriched diet, the chain elongation of 20:5n-3 might be stimulated by the enhanced chain shortening of 22:6n-3.

PA is an intermediate in glycerolipid synthesis, and is mainly produced from 1-acylglycerophosphate with microsomal fractions (47). Berge *et al*. (36) reported that the production of PA and/or LPA from glycerophosphate in either mitochondrial or microsomal fractions was higher in rats fed fish oil than in rats fed soy oil. We also found that the acylation of

LPA was more active in rats fed a DHA-enriched diet (Table 4); however, the elevated activity of PA synthesis did not seem to affect TG synthesis, considering that the PAP activity and the presumed concentration of TG calculated from the amount of total fatty acid (data not shown) were almost the same in all of the groups. Compared to a fat containing 18:3n-3 and/or 18:2n-6, a DHA-enriched fat did not have a distinct effect on TG synthesis. However, Marsh *et al*. (48) observed decreases in hepatic activities of PAP, TG hydrolase, and diacylglycerol hydrolase in rats fed fish oil compared to rats fed a commercial chow.

The amount of 22:6n-3 accumulated in the liver (*ca*. 40 mg) was much less than that consumed in a DHA-enriched diet, even though fatty acid ethyl esters are absorbed less than TG in rats (49). Fatty acid ethyl esters appear to be well absorbed in humans (50,51); however, the intake of n-3 LCPUFA ethyl esters is not routine for healthy subjects, and the amount ingested is generally limited. In addition, it should be noted that the lipid transfer protein activity in rats (52) is quite different from that in humans (53). The present study suggests that a considerable proportion of dietary 22:6n-3, in addition to 22:6n-3 released from glycerolipids, is degraded, while 22:6n-3 might be continually incorporated into newly synthesized glycerolipid at a steady low rate. These results suggest that an excess amount of 22:6n-3 is degraded when rats are fed a large amount of 22:6n-3, and an essential level of 22:6n-3 is maintained when rats are fed only a trace amount of n-3 fatty acid. The reconstitution of 22:5n-3 from retroconverted 20:5n-3 might have contributed to the lack of a complete disappearance of 22:6n-3 in biomembranes.

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Unusual Effects of Some Vegetable Oils on the Survival Time of Stroke-Prone Spontaneously Hypertensive Rats

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ABSTRACT: Preliminary experiments have shown that a diet containing 10% rapeseed oil (low-erucic acid) markedly shortens the survival time of stroke-prone spontaneously hypertensive (SHRSP) rats under 1% NaCl loading as compared with diets containing perilla oil or soybean oil. High-oleate safflower oil and high-oleate sunflower oil were found to have survival time-shortening activities comparable to that of rapeseed oil; olive oil had slightly less activity. A mixture was made of soybean oil, perilla oil, and triolein partially purified from high-oleate sunflower oil to adjust the fatty acid composition to that of rapeseed oil. The survival time of this triolein/mixed oil group was between those of the rapeseed oil and soybean oil groups. When 1% NaCl was replaced with tap water, the survival time was prolonged by ~80%. Under these conditions, the rapeseed oil and evening primrose oil shortened the survival time by ~40% as compared with n-3 fatty acid-rich perilla and fish oil; lard, soybean oil, and safflower oil with relatively high n-6/n-3 ratios shortened the survival time by roughly 10%. The observed unusual survival time-shortening activities of some vegetable oils (rapeseed, high-oleate safflower, high-oleate sunflower, olive, and evening primrose oil) may not be due to their unique fatty acid compositions, but these results suggest that these vegetable oils contain factor(s) which are detrimental to SHRSP rats.

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The stroke-prone spontaneously hypertensive (SHRSP) strain of rats develops the highest blood pressure among the commonly available rat strains and dies frequently of apoplexy particularly when NaCl is loaded. The dietary risk factors for this animal model are known to be low-protein and high-salt which are also suggested for human apoplexy. Although the genetic differences between the SHRSP strain and normotensive control strain (WKY) have not been fully defined, this animal model has been used widely for studies on essential hypertension, apoplexy, atherosclerosis, and age-related mental disorders.

Perilla seed oil from beefsteak plant *(Perilla frutescens)* has a fatty acid composition similar to that of linseed oil (~15% linoleic and ~60% α-linolenic acid) and is relatively common in east Asian countries. This oil has been shown to lengthen survival time of Donryu rats (a conventional strain) by ~10% (1) and those of SHRSP rats by ~15% (2) as compared with safflower oil (70% linoleic and <1% α-linolenic acid). We hypothesized that the observed beneficial effects of perilla oil on animal models of chronic diseases of the elderly (3) were due to its very low n-6/n-3 ratio. Rapeseed oil (~26% linoleic and ~8% α-linolenic acid) would be expected to have comparable beneficial effects because of its relatively low n-6/n-3 ratio and high oleate content. Low-erucic acid rapeseed oil, however, was found instead to shorten the survival time of SHRSP rats by >40% compared with soybean oil and perilla oil under 1% NaCl loading as drinking water (4).

Historically, a rapeseed strain containing both much smaller amounts of thyrotoxic sulfur compounds and erucic acid was selected, which was found to exhibit significantly less thyrotoxicity as expected. Later, however, the double-low rapeseed oil was reported to induce myocardial necrosis in a strain of the rat (5). After extensive studies, the incidence of myocardial necrosis was ascribed to the unique fatty acid composition of the rapeseed oil. A positive correlation with oleic and α-linolenic acid and a negative correlation with saturated fatty acids were proposed (6), and the necrosis was reproduced with a mixture consisting of soybean oil and triolein (7). Thus, the safety of double-low rapeseed oil for human use appears to have been established earlier as summarized by Dupont *et al.* (8).

However, the positive and negative correlations reported for myocardial necrosis (6) do not appear to apply to the observed shortening of survival time by these oils when fed to SHRSP rats (4), because perilla oil with similar proportions of saturated fatty acids and a greater proportion of α-linolenic acid than rapeseed oil extended the survival time of SHRSP rats significantly. The purpose of these studies was to examine if the survival time-shortening activity is due to the unique fatty acid composition of rapeseed oil, and if such an activity is seen in the absence of NaCl loading.

EXPERIMENTAL PROCEDURES

Diets and animals. A conventional basal diet containing 3.0% (w/w) oil (CE2; Clea Japan Co., Ltd., Tokyo, Japan) was made of fish meal, soybean meal, defatted powdered milk, wheat flour, corn, wheat bran, alfalfa meal, a vitamin mixture,

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Abbreviation: SHRSP, Stroke-prone spontaneously hypertensive rat strain.

and a mineral mixture. This basal diet and test oil (fat) were mixed at a weight ratio of 9 to 1. The final oil content was 12.7 wt% (29 energy percentage). Fat and oils commercially available for human use were used, except for high-erucic rapeseed oil which was not for human use; rapeseed oil (low-erucic), soybean oil, safflower oil (high-linoleate), high-oleate safflower oil, high-oleate sunflower oil, perilla oil, evening primrose oil, lard, triolein (from high-oleate sunflower oil), fish oil, and their mixtures. The vitamin E content (total tocopherol) of the basal diet was 26 mg/90 g diet, and those of fats and oils (per 10 g) were 51 mg (fish oil), 18 mg (perilla oil), 6.4 mg (safflower oil), and 2.4 mg (olive oil).

Dietary pellets containing supplemented fats or oils were prepared by Clea Japan Co., Ltd., except for the fish oil diet which was prepared in our own laboratory and contained about 40% water as compared with the pelleted diets containing 8% water. Diets with peroxide values below 10 meq/kg were served. The food in the cage was replaced every day in the case of fish oil diet, and every two days in other diets, in order to keep the peroxide values of the ingested diets below 100 meq/kg. Rats were kept in an air-conditioned room, and the temperature was kept at 23±3°C.

Genetically SHRSP rats were kindly provided by Dr. Y. Suzuki (Professor, Kinki University School of Medicine, Osaka, Japan), and those with high systolic blood pressures were mated to maintain the strain. Blood pressure was measured using equipment from Ueda Manufacturing Co., Ltd., Tokyo, Japan (Model UR5000). Litters were distributed to different dietary groups as far as possible. In preliminary experiments, the number of rats in each group was ~6, and it was increased to >10/group for confirmatory experiments. Rats were weaned to the conventional diet (CE2) at 3 wk of age, and the test diets were given from 4 wk of age. Diet and tap water or 1% NaCl solution were given *ad libitum.* The fatty acid compositions of the diets, determined as methyl esters by gas–liquid chromatography, are shown in Table 1.

Fractionation of rapeseed oil components. In efforts to separate presumed survival time-shortening factor(s) in rapeseed oil, rapeseed oil (low-erucic) was mixed with 2 vol of acetone or ethanol, the mixture was cooled to −70°C, left overnight, and the precipitate was filtered off. After evaporation of the solvent, the residue (*ca.* 0.5% of rapeseed oil as acetone-soluble and 0.7% as ethanol-soluble) was dissolved in soybean oil at a 5-fold concentration (in 1:5 the amount of the treated rapeseed oil) (acetone- or ethanol-soluble in Table 2). A methanol-soluble fraction (0.6% of oil) was obtained by treating the oil at room temperature (methanol-soluble). Saponification of the oil was performed by a conventional method, and the unsaponifiable fraction (0.33% of oil) was dissolved in soybean oil at a 2.5-fold concentration (unsaponifiable fraction). The oil (5.8 kg) dissolved in 9.5 L of hexane and 0.5 L of diethyl ether was mixed with 1 kg of silicic acid (Merck, Darmstadt, Germany). The mixture was stirred overnight, and 5.22 kg of oil was recovered from the hexane/diethyl ether fraction (silicic acid-treated).

Statistical analysis. Statistical analyses of the survival times were performed by Log-Rank and Wilcoxon's test using a computer program (9), "Statistics Made Visual™" (SAS Institute Inc., Cary, NC).

RESULTS

Effect of oils on survival time of SHRSP rats under 1% NaCl loading. At first, we tried to answer the question whether the unique fatty acid composition of the rapeseed oil is responsible for the shortening of survival time of SHRSP rats. In Experiment 1 (Table 3), a mixed oil with a fatty acid composition similar to that of rapeseed oil was prepared by mixing

TABLE 1
Fatty Acid Composition of Diets[a]

Fatty acid	Rapeseed oil	Rapeseed oil (high-erucic)	Soybean oil	Mixed oil[b]	Triolein/soybean oil	Safflower oil (high-oleate)	Sunflower oil (high-oleate)	Olive oil	Safflower oil	Perilla oil	Fish oil/soybean oil	Lard	Evening primrose oil
14:0	0.1	—[c]	0.2	0.1	0.1	0.1	0.2	0.2	0.2	0.1	1.6	1.2	0.1
16:0	6.5	5.3	11.6	7.1	9.4	6.2	7.1	11.4	8.8	7.9	13.3	22.2	8.1
16:1	0.2	0.2	0.3	0.1	0.1	0.1	0.1	0.5	0.2	0.2	2.9	2.3	0.1
18:0	1.5	1.3	3.6	2.0	3.2	2.3	3.5	3.0	2.5	1.8	2.8	10.7	1.8
18:1	55.4	16.3	24.8	58.1	51.2	69.1	66.3	65.5	10.2	20.7	20.6	39.4	13.0
18:2n-6	25.6	18.3	51.0	25.1	31.7	20.2	20.0	16.1	68.8	27.1	28.8	20.7	67.7
18:3n-6	—	—	—	—	—	—	—	—	—	—	—	—	6.9
18:3n-3	8.2	6.7	6.3	6.6	3.1	1.1	1.5	1.3	1.6	51.0	3.1	1.3	1.0
18:4n-3	—	—	—	—	—	—	—	—	—	—	0.8	—	—
20:1	1.2	5.4	0.5	0.3	0.5	0.3	0.7	0.3	0.5	0.3	1.2	0.9	0.3
20:4n-6	0.1	—	0.1	—	—	—	—	0.2	0.1	0.1	1.3	0.2	0.4
20:5n-3	0.3	0.3	0.6	0.2	0.3	0.2	0.2	0.2	0.5	0.3	5.0	0.5	0.2
22:1	0.3	45.4	—	—	—	—	—	—	—	—	—	—	—
22:6n-3	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.5	0.6	0.5	18.6	0.6	0.4

[a]A conventional diet containing 3% oil (90 g) was mixed with 10 g of lard, oil or oil mixtures, and typical fatty acid composition of the diets is shown in weight percentage. Some variations in fatty acid compositions were noted among the lots of oils used. Fatty acid composition of triolein/soybean oil/perilla oil diet used in Experiment 5 was not presented but was very similar to that of rapeseed oil. Unsaturated fatty acids with 20- and 22-carbon chains in the diets supplemented with vegetable oils were originated mainly from fish meat in the basal diet and partly from unidentified components of the oils.

[b]Diet supplemented with a mixture of 75% high-oleate safflower oil, 12.5% safflower oil, and 12.5% perilla oil.

[c]Not detectable or trace amounts.

TABLE 2
Survival Times of SHRSP Rats Fed Different Fractions of Rapeseed Oil Under NaCl Loading

Dietary group Number of animals	Rapeseed oil $n = 12$	Soybean oil $n = 12$	Acetone-soluble[a] $n = 6$	Ethanol-soluble[a] $n = 6$	Methanol-soluble[a] $n = 6$	Unsaponifiable fraction[a] $n = 7$	Silicic acid-treated[a] $n = 9$
Survived day ± SD (survival rate)	104 ± 6	213 ± 98	>202 ± 17[b] (2:6)	>172 ± 28[b] (1:6)	>200 ± 34[b] (3:6)	>152 ± 10[b] (7:7)	113 ± 6

[a]See text for the preparation of these fractions from rapeseed oil.
[b]Experiments were stopped when the difference vs. rapeseed oil group became statistically significant, and the numbers of rats surviving/total rats when experiments were stopped are shown in parentheses. SHRSP: stroke-prone spontaneously hypertensive rat strain.

TABLE 3
Survival Times of SHRSP Rats Fed Different Oils Under 1% NaCl Loading[a]

	Dietary group and number of animals			
Experiment 1	Rapeseed oil (Rap), $n = 6$	Soybean oil (Soy), $n = 6$	Mixed oil (high-oleate safflower/safflower/perilla) $n = 6$	
Survived day (± SD)	146 ± 12	267 ± 5	144 ± 7	
P value by Log-rank		0.001 vs. Rap	0.900 vs. Rap	
Wilcoxon	0.001 vs. Rap	0.633 vs. Rap		
Log-rank		0.001 vs. Soy		
Wilcoxon			0.001 vs. Soy	
Experiment 2	Rapeseed oil (Rap), $n = 12$	Soybean oil (Soy), $n = 11$	High-oleate safflower oil, $n = 12$	
Survived day (± SD)	122 ± 5	235 ± 17	116 ± 5	
P value by Log-rank		0.000 vs. Rap	0.243 vs. Rap	
Wilcoxon		0.000 vs. Rap	0.153 vs. Rap	
Log-rank			0.000 vs. Soy	
Wilcoxon			0.000 vs. Soy	
Experiment 3	Rapeseed oil (Rap), $n = 6$	Soybean oil (Soy), $n = 6$	High-oleate sunflower oil (HOS), $n = 8$	High-erucic rapeseed oil, $n = 8$
Survived day (± SD)	136 ± 11	189 ± 14	135 ± 7	115 ± 4
P value by Log-rank		0.005 vs. Rap	0.940 vs. Rap	0.024 vs. Rap
Wilcoxon		0.006 vs. Rap	0.898 vs. Rap	0.045 vs. Rap
Log-rank			0.008 vs. Soy	0.000 vs. Soy
Wilcoxon			0.013 vs. Soy	0.001 vs. Soy
Log-rank				0.018 vs. HOS
Wilcoxon				0.035 vs. HOS
Experiment 4	Rapeseed oil (Rap), $n = 6$	Soybean oil (Soy), $n = 6$	Safflower oil (Saf), $n = 7$	Triolein/soybean oil (Tri), $n = 6$
Survived day (± SD)	132 ± 11	241 ± 33	204 ± 19	172 ± 19
P value by Log-rank		0.005 vs. Rap	0.004 vs. Rap	0.047 vs. Rap
Wilcoxon		0.006 vs. Rap	0.005 vs. Rap	0.071 vs. Rap
Log-rank			0.151 vs. Soy	0.046 vs. Soy
Wilcoxon			0.350 vs. Soy	0.102 vs. Soy
Log-rank				0.223 vs. Saf
Wilcoxon				0.248 vs. Saf
Experiment 5	Rapeseed oil (Rap), $n = 11$	Soybean oil (Soy), $n = 11$	Olive oil (Oli), $n = 12$	Triolein/soybean oil/perilla oil, $n = 12$
Survived day (± SD)	131 ± 7	212 ± 25	155 ± 19	172 ± 20
P value by Log-rank		0.004 vs. Rap	0.401 vs. Rap	0.096 vs. Rap
Wilcoxon		0.015 vs. Rap	0.854 vs. Rap	0.161 vs. Rap
Log-rank			0.015 vs. Soy	0.388 vs. Soy
Wilcoxon			0.035 vs. Soy	0.326 vs. Soy
Log-rank				0.434 vs. Oli
Wilcoxon				0.312 vs. Oli

[a]Litters of SHRSP rats (male) were distributed among different dietary groups and the test diets were fed from 4 wk of age. See Table 2 for other abbreviations.

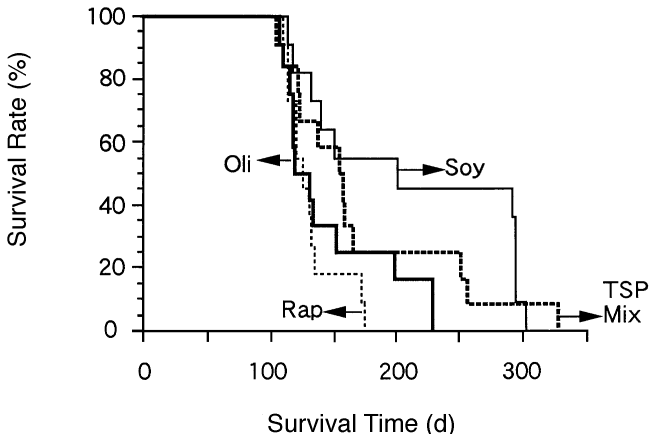

FIG. 1. Effect of vegetable oils on survival rate of stroke-prone spontaneously hypertensive (SHRSP) rats in the presence of 1% NaCl loading. A conventional basal diet containing 3% oil and test oil were mixed at a weight ratio of 9:1, and pelleted diet was fed to SHRSP rats from 4 wk of age. NaCl solution (1%) was given *ad libitum* as drinking water. Rapeseed oil (Rap, $n = 11$), olive oil (Oli, $n = 12$), soybean oil (Soy, $n = 11$), and triolein/soybean oil/perilla oil mixture (TSP Mix, $n = 12$) were examined. See Experiment 5 in Table 2 for detail of statistical analyses.

high-oleate safflower oil (75%), perilla oil (12.5%), and safflower oil (12.5%). The mixed oil was found to shorten the survival time by 45% compared to soybean oil similar to rapeseed oil. Earlier, we noted that perilla oil enhanced and safflower oil shortened the survival time of SHRSP rats by ~10% (1,2) compared with soybean oil. We suspected that the high-oleate safflower oil in the mixed oil was mainly responsible for the survival time-shortening activity of the mixed oil, since the high-oleate safflower oil was found to shorten the survival time similarly to the rapeseed oil (Experiment 2, Table 3). High-oleic sunflower oil was as effective as rapeseed oil, and high-erucic rapeseed oil reduced the survival time even more (Experiment 3). All the high-oleate vegetable oils examined (Experiments 1–3) were found to reduce the life expectancy of SHRSP rats.

When soybean oil and commercially available triolein, prepared from high-oleate sunflower oil, were mixed to adjust the oleate content to that of rapeseed oil, the linoleate content (32%) and α-linolenate content (3%) were slightly different from those of rapeseed oil (26% linoleate and 8% α-linolenate). The survival time of the triolein/soybean oil group was between those of the rapeseed oil and soybean oil groups (Experiment 4). The survival time of the safflower oil group was significantly less than that of the soybean oil group, as reported earlier (2). Because the number of animals was relatively small in Experiment 4, a similar experiment was performed using increased numbers of animals in Experiment 5 to test a triolein/soybean oil/perilla oil mixture. The survival times relative to that of soybean oil group (100) were 78% (triolein/soybean oil/perilla oil group), 72% (olive oil group), and 61% (rapeseed oil group) (Fig.1). The triolein/soybean oil/perilla oil mixture had almost the same fatty acid composition as that of rapeseed oil, but the survival time of this group tended to be longer than that of the rapeseed oil group, although the difference was not statistically significant.

In Experiment 5, the systolic blood pressure and pulse rate were measured at 4 and 8 wk of age and no significant differences among the dietary groups were detected (Table 4).

Effect of lard, vegetable oils, and a docosahexaenoic acid-rich fish oil on survival time of SHRSP rats in the absence of 1% NaCl loading. The survival times increased approximately by 80% when 1% NaCl solution was replaced with tap water. However, the survival time of the rapeseed oil group remained less, i.e., 61% of the soybean oil group (Experiment 6 in Table 5, Fig.2). Fatty acid composition of the evening primrose oil was much different from that of high-oleate vegetable oils (Table 1), but the survival time of this group was almost the same as that of the rapeseed oil group. The survival times of the perilla oil group and fish oil group were longer than that of the soybean oil group by 7 and 9%, respectively, but these differences were not statistically significant. In a similar experiment (Experiment 7), lard and safflower oil were found not to have such a marked survival time-shortening activity as observed with rapeseed oil, evening primrose oil, and other high-oleate vegetable oils (Table 3). Again, n-3 fatty acid-rich oils (fish oil and perilla oil) were beneficial for the survival of SHRSP rats as compared with lard and all other vegetable oils examined. The docosahexaenoate-rich fish oil, supplemented with soybean oil to meet the requirement for linoleate, gave the highest survival time.

Some attempts to separate the presumed survival time-shortening factor. Several fractions were prepared from rapeseed oil and tested to see if survival factor(s) could be removed

TABLE 4
Effect of Dietary Oils on Systolic Blood Pressure and Pulse Rate of SHRSP Rats under 1% NaCl Loading

	Dietary group[a] and number of animals			
	Rapeseed oil ($n = 11$)	Soybean oil ($n = 11$)	Olive oil ($n = 12$)	Triolein/soybean oil/perilla oil ($n = 12$)
At 4 wk of age				
Systolic blood pressure (mmHg)	157 ± 20	156 ± 23	154 ± 17	161 ± 21
Pulse rate (beat/min)	457 ± 75	431 ± 75	436 ± 70	416 ± 84
At 8 wk of age				
Systolic blood pressure (mmHg)	197 ± 16	198 ± 15	208 ± 15	192 ± 17
Pulse rate (beat/min)	382 ± 54	378 ± 36	366 ± 46	382 ± 45

[a]These dietary groups correspond to Experiment 5 in Table 2. See Table 2 for abbreviation.

TABLE 5
Survival Times of SHRSP Rats Fed Lard or Different oils Without NaCl Load

	Dietary group and number of animals				
Experiment 6	Rapeseed oil (Rap), $n = 12$	Evening primrose oil (Epo), $n = 12$	Soybean oil (Soy), $n = 11$	Perilla oil (Per), $n = 12$	Fish/soybean oil (Fis), $n = 12$
Survived day (± SD)	254 ± 12	261 ± 19	416 ± 16	445 ± 15	453 ± 16
P value by Log-rank		0.227 vs. Rap	0.000 vs. Rap	0.000 vs. Rap	0.000 vs. Rap
Wilcoxon		0.455 vs. Rap	0.000 vs. Rap	0.000 vs. Rap	0.000 vs. Rap
Log-rank			0.000 vs. Epo	0.000 vs. Epo	0.000 vs. Epo
Wilcoxon			0.000 vs. Epo	0.000 vs. Epo	0.000 vs. Epo
Log-rank				0.178 vs. Soy	0.078 vs. Soy
Wilcoxon				0.204 vs. Soy	0.106 vs. Soy
Log-rank					0.589 vs. Per
Wilcoxon					0.819 vs. Per
	Lard	Safflower oil	Perilla oil	Fish/soybean oil	
Experiment 7	$n = 11$	(Saf), $n = 11$	(Per), $n = 11$	(Fis), $n = 11$	
Survived day (± SD)	412 ± 10	404 ± 15	444 ± 13	488 ± 17	
P value by Log-rank		0.5854 vs. Lard	0.0219 vs. Lard	0.0006 vs. Lard	
Wilcoxon		0.8189 vs. Lard	0.0834 vs. Lard	0.0033 vs. Lard	
Log-rank			0.0217 vs. Saf	0.0010 vs. Saf	
Wilcoxon			0.0493 vs. Saf	0.0039 vs. Saf	
Log-rank				0.0153 vs. Per	
Wilcoxon				0.0545 vs. Per	

or isolated from the oil. Rapeseed oil was treated overnight at −70°C with acetone or ethanol, and the soluble fractions were mixed with soybean oil at a 5-fold concentration (in 1:5 the amount of the treated rapeseed oil). These fractions (acetone-soluble and ethanol-soluble in Table 2) did not shorten the survival time significantly. Similarly, methanol extracts of rapeseed oil (methanol-soluble), obtained at a room temperature, did not exhibit the survival time-shortening activity. Unsaponifiable materials from rapeseed oil were mixed with soybean oil at a 2.5-fold concentration (unsaponifiable fraction), and were found not to exhibit the activity.

Rapeseed oil (5.8 kg) in 9.5 L of hexane and 0.5 L of diethyl ether were mixed with 1 kg of silicic acid. The mixture was stirred overnight, and 5.22 kg of oil was recovered in the hexane/diethyl ether layer (silicic acid-treated). The survival time-shortening activity could not be removed. These experiments suggest that the survival time-shortening factor in rapeseed oil is relatively lipophilic, nonpolar in nature, and is closely associated with the triacylglycerols of the oil.

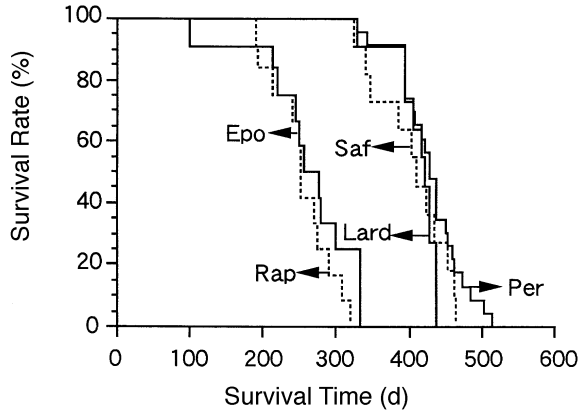

FIG. 2. Effect of vegetable oils on survival rate of stroke-prone spontaneously hypertensive (SHRSP) rats in the absence of 1% NaCl loading. A conventional basal diet containing 3% oil and test oil (lard) were mixed at a weight ratio of 9:1, and pelleted diet was fed to SHRSP rats from 4 wk of age, except for fish oil/soybean oil diet which was prepared in our laboratory (see text for details). Rapeseed oil (Rap, $n = 12$), evening primrose oil (Epo, $n = 12$), safflower oil (Saf, $n = 11$), perilla oil (Per, $n = 23$), or lard (Lard, $n = 11$) were examined. Tap water was given *ad libitum* as drinking water. See Table 4 for details of statistical analyses.

DISCUSSION

The survival time-shortening activity (4) was found not to be confined to rapeseed oil; high-oleate safflower oil, high-oleate sunflower oil, evening primrose oil, and olive oil exhibited comparable activities. Oleate esters have been implicated in certain disease conditions such as atherosclerotic plaques where cholesterol oleate accumulated as the major molecular species (10). However, in this case a high-oleate content itself does not appear to be crucial for this animal model because lard and a microbial oil containing 36–39% oleic acid were relatively safe, while evening primrose oil, containing only 13% oleic acid, was detrimental (Ref. 4 and Table 5). The survival time of the group fed a triolein/soybean oil mixture with an oleate content similar to that of rapeseed oil was between those fed soybean oil and rapeseed oil (Experiment 4 in Table 3). Similarly, the survival time of the group fed the triolein/soybean oil/perilla oil mixture, with a fatty acid composition very similar to that of rapeseed oil, was also found to be between those fed soybean oil and rapeseed oil, although the difference between the mixed oil group and rapeseed oil group was not statistically significant (Experiment 5 in

Table 3). Based on these results, it appears unlikely that the observed survival time-shortening activity is due to the unique fatty acid compositions of these oils. However, it should be noted that the triolein used to make up the two mixed oils was derived from high-oleate sunflower oil. If we presume that rapeseed oil and the other high-oleate vegetable oils contain detrimental factor(s) for SHRSP rats, then the factor(s) may have partially carried over from these oils through the purification steps.

Minor components other than triacylglycerol in high-oleic acid vegetable oils, such as olive oil and high-oleate safflower oil, have been implicated in a number of studies. A high-oleate safflower oil was shown to suppress mammary and colorectal carcinogenesis compared to high-linoleate safflower oil (11), whereas both olive oil and safflower oil were reported to stimulate colorectal carcinogenesis in contrast to perilla oil (12,13). Various preparations of olive oil are known to differ in their contents of minor components and the one used by Onogi *et al.* (12) might have contained a larger quantity of minor components which stimulated carcinogenesis, e.g., β-carotene which was recently shown in clinical trials to increase the incidence of certain types of cancers (14). In fact, olive oil had been shown earlier to suppress colon carcinogenesis (15).

If physiologically active minor components are present in some vegetable oils, they are lipophilic in nature and could affect the central nervous functions after being delivered to the brain through the blood brain barrier. In fact, we found unusual behavioral patterns in rapeseed oil-fed mice as compared with those fed several other kinds of vegetable oils. The phenomena could not be accounted for simply by the differences in the fatty acid compositions, or ratio of linoleate to α-linolenate (16). We have no evidence so far for the interpretation that the presumed survival time-shortening factor also affected the behavior of mice. However, some of the results of behavioral studies in rodents fed different vegetable oils are apparently inconsistent among different laboratories (17–19). The vegetable oils with significant survival-time shortening activities were fed to animals in some behavioral studies: rapeseed oil (18,20–22) and olive oil (17). Apparent discrepancies reported for the effects of vegetable oils on behavior of rodents may have originated, at least in part, from differences in minor components other than fatty acids in the oils.

Compared to Danish people, the incidence of thrombotic diseases in native Greenlanders (Inuit) was less than one-tenth, but that of apoplexy was slightly higher (23,24). Therefore, a long-term ingestion of large amounts of fish oil was suspected to be a risk factor for apoplexy. However, this has not been confirmed by epidemiologic studies (25); although n-3 fatty acid supplementation lengthens bleeding time, there is no evidence to suggest that it causes clinically significant bleeding episodes (25). Consistently, our present (Table 5) and previous studies (2,26) have shown that long-term feedings of fish oil and perilla oil at 10 w/w% of diet were beneficial for the suppression of the development of apoplexy in SHRSP rats.

Rapeseed oil (double-low) has been recently evaluated for possible nutritional benefits associated with its relatively low n-6/n-3 ratio, high-oleate content, and low level of saturated fatty acids. In fact, dietary rapeseed oil and olive oil resulted in decreased n-6 fatty acids with concomitant increases in oleic and n-3 fatty acids, which proved to be very effective for the secondary prevention of coronary heart disease (27). However, the observed beneficial effect of the combination of rapeseed oil and olive oil on coronary heart disease is interpreted to be due to their relatively low linoleic acid contents and n-6/n-3 ratios (28). We need to solve the problem of the survival-time shortening activity observed in SHRSP rats before advising people to consume a large amount of rapeseed oil and olive oil. At present, we need to evaluate carefully the potential merits and problems of high-oleate vegetable oils, including rapeseed oil, in human nutrition.

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Oral Acetylsalicylic Acid Induces Biliary Cholesterol Secretion in the Rat

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ABSTRACT: Several agents can alter biliary cholesterol secretion, critical for cholesterol excretion and gallstone formation. Although salicylate effects on bile formation and gallstones have been studied, biliary lipid secretion has not been measured during oral aspirin treatment. We examined whether oral acetylsalicylic acid affects bile lipid secretion. Three groups of young rats were fed chow for 3 wk. Two of the groups then received aspirin at either 1.67 or 3.33 g/kg diet for 4 d. Serum, hepatic, and bile lipids were measured, as were enzymes of cholesterol synthesis and esterification. With oral aspirin, bile cholesterol secretion increased by 42% and hepatic cholesteryl ester content decreased by 40%. Serum cholesterol and hepatic free cholesterol did not change. To evaluate mechanisms of the cholesterol hypersecretion, hypothyroid animals fed low-fat or fish oil diets and repleted with triiodothyronine were also studied. Aspirin stimulated cholesterol secretion to a degree similar to triiodothyronine. An additive response was seen in fish oil-fed rats. Aspirin did not appear to have a primary action on 3-hydroxy-3-methylglutaryl-CoA reductase or acyl CoA:cholesterol acyltransferase activities, and had no direct effect on esterification of cholesterol by isolated hepatocytes. Aspirin may directly increase cholesterol transport into bile or have cell membrane effects which alter cholesterol transport. It remains to be determined whether the observed alterations in bile cholesterol secretion are specific to the rat or also apply to humans. *Lipids 32*, 753–758 (1997).

Several drugs can influence bile flow and bile lipid secretion. Substances which alter biliary cholesterol secretion usually have a more general impact on cholesterol metabolism, particularly cholesterol synthesis. Inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase block the regulatory enzyme of cholesterol synthesis (1). In humans given the reductase inhibitor lovastatin, cholesterol synthesis and cholesterol secretion into bile were reduced (2,3). In the rat, lovastatin also acutely reduced bile cholesterol (4). However, reductase inhibitors given long-term also induce the amount of hepatic enzyme protein, especially in rats (1). Thus, rats withdrawn from inhibitor increased cholesterol synthesis fivefold and bile sterol secretion threefold (1).

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Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; ASA, acetylsalicylic acid; FO, fish oil; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; LF, low-fat diet; SA, salicylic acid; T3, triiodothyronine.

Bile cholesterol secretion was also increased by progesterone, an inhibitor of cholesterol esterification *via* acyl CoA:cholesterol acyltransferase (ACAT), and by diosgenin, which impairs cholesterol absorption, increases synthesis, and reduces esterification (4,5). Thyroid hormone has many actions on cholesterol metabolism (6–8). Bile flow and cholesterol secretion were reduced in hypothyroid rats, while triiodothyronine (T3) repletion rapidly caused hypersecretion (7,8). Increased hepatic HMG-CoA reductase activity followed bile cholesterol hypersecretion.

In rats, dietary fish oil lowered serum cholesterol and increased bile cholesterol secretion (9–11). The extent of increase varied with experimental conditions. Secretion increased 50% from an acute bile fistula, and 400% from chronic bile-diverted animals. Fish oil (FO) n-3 fatty acids inhibit very low density lipoprotein formation (12) and increase low density lipoprotein receptor activity (13), actions which could increase bile cholesterol secretion.

Some organic anions excreted into bile also affect bile lipid secretion. Iodipamide, ampicillin, and bilirubin reduced bile lecithin and cholesterol secretion in rats to 25% of basal, without altering bile salt output (14,15). The former two agents also increased bile flow. The action of bilirubin on lipid secretion was seen only when conjugated bilirubin was transported into bile (16).

Infusion of sodium salicylate (SA) or lysine acetylsalicylate (ASA) increased bile flow in dogs and monkeys (17–19). Bile salt secretion did not change in dogs given the salicylates (17,19), but lecithin and cholesterol secretion fell markedly in those given lysine ASA (19). Bile lipid secretion was not altered in monkeys infused with sodium SA (18). As a cyclooxygenase inhibitor, ASA has been suggested to inhibit cholesterol crystal nucleation in gallbladder bile. Sodium ASA treatment did not alter bile cholesterol saturation index in humans (20), but bile lipid secretion was not measured. In rat studies, we report that chronic oral ASA stimulates bile cholesterol secretion and, to a lesser extent, secretion of lecithin and bile salt.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 75–100 g were fed a low-fat diet (LF) prepared from ground stock rodent chow of

Ralston Purina (St. Louis, MO) as previously described (21). Animals were divided into three groups and fed LF for 3–4 wk. Group one served as control, while groups two and three received 1.67 g/kg and 3.33 g/kg ASA admixed into LF fed for 4 d prior to sacrifice. These animals were given regular water and were maintained in a light-reversed room (dark 0700–1900 h; light 1900–0700 h).

At time of study, animals were weighed and anesthetized with sodium pentobarbital (Nembutal) 60 mg/kg i.p. The bile duct was then cannulated with PE-10 tubing and bile was collected for 2 h, weighed to determine volume, and frozen. Animals were then exsanguinated and the serum was frozen. The liver was removed, rinsed in iced saline, blotted, and weighed. A 1-g sample was promptly homogenized in 11 mL iced 0.25 M sucrose using a Polytron with 10 mm probe at setting 3–4 for 5 s. Homogenate samples were aliquoted for protein analysis and lipid extraction. Remaining homogenate was used to prepare microsomes. Microsomal HMG-CoA reductase activity was measured as described (7). Microsomal ACAT activity was measured using endogenous cholesterol, as we have reported for kidney, with incubation time reduced to 5 min (22).

Total and free cholesterol of liver homogenates and microsomes, and total cholesterol of bile and serum were extracted and quantified by gas–liquid chromatography (GLC) as described (7). Total bile acids were measured using the methods of Talalay (23). Bile lecithin was measured using the PL Kit-K from Nippon-Shoji Kaisha, Ltd. (Osaka, Japan) (24). Protein was determined by the method of Lowry *et al.* (25). Bile was directly transesterified, and liver homogenate phospholipids were extracted and transesterified. Fatty acid methyl esters of the phospholipids were measured by capillary GLC (21).

Serum and bile SA levels were determined by GLC of the methyl derivative. A 0.2-mL sample of serum or bile was saturated with 80 mg NaCl in a 10×75 mm culture tube. An internal standard of 2 μg 13:0 fatty acid and 50 μg of the antioxidant butylated hydroxytoluene in 0.1-mL MeOH was then added, followed by 15 μL of 7 M H_3PO_4 and 1 mL diethyl ether. Tubes were capped, vortexed, and centrifuged. The ether phase was transferred to a conical vial. Ether was removed with nitrogen and traces of water with anhydrous ethanol. Residue was dissolved in 100 μL of 10% methanol in diethyl ether and methylated by addition of diazomethane at −70°C. GLC analysis was performed as described for fatty acid methyl esters (21). Diazomethane was prepared using a glassware kit and the precursor *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide from Aldrich (Milwaukee, WI).

In a second experiment, similar rats were fed LF diet or LF supplemented with 10% (w/w) fish oil (FO). Cholesterol was added to LF to compensate for cholesterol present in FO, so that both diets contained 0.1 mg cholesterol per kcal (21). These rats were made hypothyroid by adding methimazole 0.025% to drinking water (7). Four days prior to study, each diet group was further divided into four groups: one group remained hypothyroid, a second group received 200 μg T3 per 100 g body weight i.p. 48 h prior to study (7), a third group had ASA added to the diet at 1.67 g/kg, and the fourth group received ASA diet plus T3.

Additional animals were prepared to study effects of ASA and SA on esterification of cell membrane free cholesterol. Isolated liver cells, from animals fed LF alone or LF containing 1.67 g/kg ASA, were prepared using 0.5 mM EGTA (26,27). Yields were 400×10^6 hepatocytes per liver and viability was 90%. Cells were diluted to 5.5×10^6/mL and preincubated for 90 min at 4°C to label membranes with 1 μCi [^{3}H] cholesterol/mL (22.5 Ci/mmol), added as a 1 μCi/μL ethanol solution. Cells were centrifuged and resuspended in unlabeled media. One-mL aliquots of cell suspension were placed in 25-mL polycarbonate Erlenmeyer flasks containing 4 mL media continuously gassed with (95%O_2-5%CO_2), with or without 100 μg/mL ASA or 77 μg/mL SA. Cells were incubated for 4 h at 37°C, and lipids were extracted and fractionated (22). [^{3}H]cholesterol esters were measured as the fraction of total [^{3}H] cholesterol.

This project was approved by the Animal Use Subcommittee of the Minneapolis VA Medical Center (Minneapolis, MN). All animals were handled in accordance with all applicable local and federal regulations concerning laboratory animals.

Statistics. Values reported are mean ± SD. Student's unpaired *t*-test was used to calculate significance.

RESULTS

Table 1 shows weight, bile lipid secretion rate, serum and liver cholesterol level, and activities of HMG-CoA reductase and ACAT in control and ASA-fed euthyroid animals. No significant differences were seen for body or liver weights, serum cholesterol, or bile flow between ASA-treated and control animals. Bile cholesterol secretion, however, was significantly higher for animals given ASA, while biliary secretion of phospholipid and bile salts increased only modestly. Bile cholesterol saturation index did not change significantly in either ASA-fed groups.

No significant changes occurred in the fatty acid composition of whole liver or bile phospholipids as a result of ASA treatment (data not shown). Total cholesterol content of liver homogenate was similar for all groups, but ASA treatment decreased hepatic cholesterol ester content significantly to 60% that of control animals. Microsomal total and ester cholesterol contents were significantly reduced by the high ASA dose. Consistent with this finding, HMG-CoA reductase activity was significantly elevated above control values in the high ASA dose animals. ACAT activity was unaffected.

To exclude a direct effect of ASA on measured HMG-CoA reductase or ACAT activity, enzyme activities were assayed with added ASA in ethanol or sodium SA in water. At concentrations between 3 and 100 μg/mL, no effect on measured enzyme activity was produced by these compounds (Table 2). Serum salicylic acid levels in high- and low-dose ASA-fed animals averaged 81 and 45 μg/mL, respectively, while bile contained 16 and 12 μg/mL. These serum values are within the human therapeutic range.

TABLE 1
Effect of Acetylsalicylic Acid (ASA) on Parameters Measured in the Rat[a]

Parameter	Low-fat diet (LF) (n = 10)	LF + ASA 1.67 (g/kg) (n = 11)	LF + ASA 3.33 (g/kg) (n = 10)
Animal weight (g)	231 ± 23	236 ± 13	224 ± 11
Liver weight (g)	10.0 ± 1.0	9.7 ± 1.3	9.1 ± 0.7
Serum cholesterol (mM)	2.19 ± 0.37	2.14 ± 0.24	2.15 ± 0.13
Bile			
Flow (mL/kg/h)	4.56 ± 0.94	4.71 ± 0.77	4.68 ± 0.77
Cholesterol (μmol/kg/h)	2.79 ± 0.61	4.02 ± 1.27*	3.91 ± 1.39*
Phosphatidylcholine (μmol/kg/h)	17.9 ± 3.6	21.8 ± 5.2	23.7 ± 5.1*
Total bile acids (μmol/kg/h)	116 ± 42	153 ± 34*	142 ± 26
Saturation index	0.67 ± 0.11	0.70 ± 0.12	0.74 ± 0.13
Liver cholesterol			
Whole: total	31.1 ± 2.5	29.5 ± 2.3	29.7 ± 1.9
ester	3.6 ± 1.2	2.1 ± 1.5*	2.2 ± 1.2*
Microsomal: total	49.7 ± 5.3	48.5 ± 7.6	43.7 ± 6.1*
ester (μmol/g protein)	2.7 ± 1.5	2.9 ± 0.8	1.6 ± 0.7*
Liver microsomal			
HMG-CoA reductase	199 ± 67	268 ± 90	368 ± 137*
ACAT (pmol/mg protein/min)	542 ± 83	499 ± 116	513 ± 171

[a]Values are means ± SD. HMG, 3-hydroxy-3-methylglutaryl. ACAT, acyl-CoA:cholesterol acyltransferase. *Difference between LF and LF + ASA, $P < 0.05$.

A second group of experimental animals was prepared in order to evaluate effects of ASA on biliary lipid secretion changes which we have shown to be associated with dietary FO and T3 status (7,21). Results are shown in Figure 1. Bile cholesterol secretion in hypothyroid rats fed LF or FO, rats repleted with T3, rats given oral ASA, and rats given the combination of T3 repletion plus ASA are shown. There was no difference between LF or FO diet for bile cholesterol secretion by hypothyroid animals. Thyroid hormone repletion significantly increased biliary cholesterol secretion in both diet groups, as previously shown (7,21). Bile cholesterol secretion also increased, to an extent equivalent to that seen with T3, in hypothyroid animals receiving only low-dose dietary ASA. In fact, the ASA effect in FO-fed rats was greater than the T3 effect in LF rats. The combination of T3 repletion plus dietary ASA was similar to that of either agent alone in the LF group. However in FO-fed rats, T3 repletion plus ASA treatment had an additive effect on biliary cholesterol secretion.

As seen for euthyroid rats, microsomal HMG-CoA reductase activity increased in both groups of hypothyroid rats given ASA. HMG-CoA reductase activity was slightly lower in hypothyroid LF than in FO animals, but values nearly doubled in all animals given ASA alone, or T3 plus ASA in combination (Table 3). In these hypothyroid animal studies, elevated baseline serum cholesterol values decreased with all interventions. Serum cholesterol levels of hypothyroid animals on either diet were 2.4 mM. Values fell significantly to 1.7 mM following either ASA feeding, T3 repletion, or the combination of T3 plus ASA. Serum SA levels in hypothyroid animals were less than half the values seen in euthyroid rats, reflecting reduced diet consumption by hypothyroid animals.

Cholesterol esterification was studied in isolated hepatocyte preparations from a third group of LF or LF plus ASA-fed animals. Cholesterol esters formed by hepatocytes prelabeled with [^{3}H] cholesterol and then incubated in media alone or media containing ASA or SA are shown in Table 4. Presence of ASA or SA had no direct effect on cholesterol esterification by hepatocytes of animals fed either diet. However, ASA prefeeding significantly increased hepatocyte cholesterol ester formation above that of control LF diet.

TABLE 2
Enzyme Activity in Control Rat Hepatic Microsomes Incubated with and without ASA and Salicylic Acid (SA)[a]

Drug addition	HMG-CoA reductase (as percentage of 0 addition)	ACAT (as percentage of 0 addition)
ASA (μg/mL)		
0	100	100
3	101	94
10	95	93
30	94	93
100	99	98
NaSA (μg/mL)		
3	93	87
10	93	99
30	96	101
100	95	102

[a]HMG-CoA reductase and ACAT assays were performed without (0) or with (3, 10, 30, 100 μg/mL) addition of salicylates directly to the reaction mixture. No direct effect seen. See Table 1 for other abbreviations.

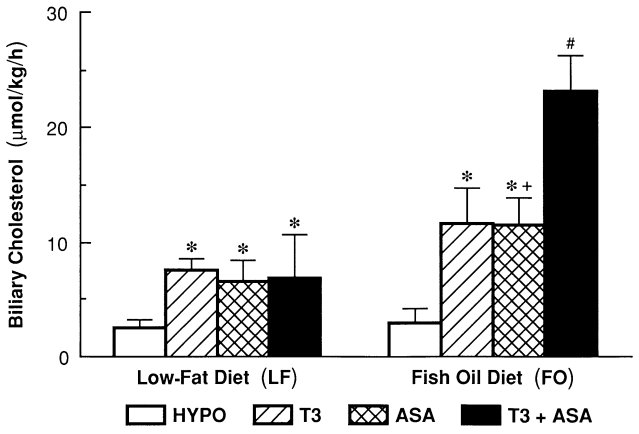

FIG. 1. Hypothyroid rats consuming LF or FO diet were administered triiodothyronine (T3), acetylsalicylic acid (ASA), or T3 plus ASA. Biliary cholesterol secretion was measured in control hypothyroid animals on each diet, and after each intervention. * Values different from LF and FO, $P < 0.05$. + Values, different from LF given T3, $P < 0.05$. # Values different from all other groups, $P < 0.05$.

TABLE 3
HMG-CoA Reductase Activity in Hypothyroid Rats and Hypothyroid Rats Given ASA or ASA + T3 (animals shown in Fig. 1)[a]

Animal diets	Low-fat diet (LF)	Fish-oil diet (FO)
Treatment	Liver microsomal HMG-CoA reductase (pmol/mg protein/min)	
Hypo	183 ± 122 ($n = 6$)	328 ± 37 ($n = 6$)
Hypo + ASA	560 ± 28* ($n = 2$)	549 ± 35* ($n = 2$)
Hypo + ASA + T3	617 ± 280* ($n = 2$)	587 ± 49* ($n = 2$)

[a]Values are means ± SD. * = Different from Hypo alone, $P < 0.05$. T3, triiodothyronine. See Table 1 for other abbreviations.

DISCUSSION

Under most conditions, bile cholesterol and phospholipid secretion are coupled to bile acid secretion (28–30). The mechanism(s) for this linkage are uncertain. It is also postulated that a specific intracellular free cholesterol pool is available for biliary secretion: a pool which can be expanded by synthesis or reduced by ester formation, with concomitant bile secretion change (4,5,31).

Previous studies have evaluated effects of intravenous salicylates on bile and bile lipid secretion. Salicylate infusion produced choleresis and reduced phospholipid and cholesterol secretion into bile (19). It was postulated that dilution of bile acids impaired their ability to extract phospholipid and cholesterol from canalicular membranes. However, the acute intravenous infusion model has limited experimental applicability for studying salicylate effects.

Oral aspirin and salicylates also have been studied to a limited extent in regard to gallstone formation. Aspirin appeared to prevent gallstones in prairie dogs fed a lithogenic diet (32). In humans, 600 mg sodium ASA four times daily, alone or with chenodeoxycholate, produced no ASA-specific change in bile cholesterol saturation index (20). Low-dose oral ASA also did not alter bile saturation index in obese subjects losing weight (33). However, bile lipid secretion was not measured in these studies.

The current studies show increased bile cholesterol secretion in young rats given oral ASA, at blood levels typical for human ASA use. In spite of the fact that bile cholesterol secretion was augmented by ASA treatment, bile cholesterol saturation index did not change significantly. This resulted from the fact that phospholipid and bile acid secretion also increased after ASA treatment, though to a lesser extent than cholesterol secretion. Previous studies also showed no effect of ASA on saturation index (20,33).

Consistent with the augmented bile cholesterol secretion were the observations that HMG-CoA reductase activity increased at high ASA dose, and the finding of diminished hepatic cholesterol esters in ASA-treated rats. Reductase activity usually reflects cholesterol synthesis. Since phospholipid and bile acid secretion also increased during ASA treatment, it seems unlikely that ASA had a primary effect on cholesterol synthesis. We also failed to find direct effects of ASA or SA on reductase or ACAT activity. It seems likely that the increased cholesterol secretion in ASA-treated rats leads to the diminished microsomal cholesterol and whole liver cholesterol ester content and the increased cholesterol synthesis. This is consistent with prior evidence for a hepatocyte free cholesterol pool which metabolically interrelates cholesterol synthesis, biliary secretion, and esterification (4,5,31).

In the second set of experiments, ASA augmented bile lipid secretion by LF-fed hypothyroid rats to the same extent as thyroid hormone repletion (7,8,21). Further cholesterol hypersecretion was seen after ASA treatment of hypothyroid

TABLE 4
Effect of ASA and Salicylic Acid (SA) on Cholesterol Ester Formation from Hepatocyte Membranes Pre-labeled with [3H]Cholesterol[a]

Animal diets	LF			LF + ASA 1.67 (g/kg)	
Hepatocytes (cultures)	Control ($n = 12$)	ASA 100 μg/mL ($n = 11$)	SA 77 μg/mL ($n = 10$)	Control ($n = 12$)	ASA 100 μg/mL ($n = 8$)
Percentage ester*	1.40 ± 0.35	1.39 ± 0.31	1.29 ± 0.25	4.29 ± 2.05[a]	3.56 ± 2.69[b]

[a]Expressed as percentage of total cell [^{3}H]cholesterol, following 4 h incubation at 37°C. Values are mean ± SD. a = Difference between LF control and ASA-fed, $P < 0.01$. b = Difference between LF control/ASA incubated and ASA-fed/ASA-incubated, $P < 0.02$. See Tables 1 and 3 for other abbreviation.

TABLE 5
Effects of Aspirin upon Hepatic Cholesterol Metabolism in the Rat Are Not Dependent upon the Surgical Intervention (2-h) (for bile drainage)[a]

Treatment	HMG-CoA reductase (pmol/mg protein/min) Microsomal	Hepatic cholesterol ester (μmol/g protein) Whole	Microsomal
LF unoperated ($n = 4$)	273 ± 39	9.2 ± 1.7	7.7 ± 0.8
LF operated/drained ($n = 5$)	234 ± 95	9.3 ± 3.2	6.9 ± 0.9
LF + ASA unoperated ($n = 5$)	487 ± 67*	3.3 ± 0.4*	2.7 ± 0.4*
LF + ASA operated/drained ($n = 5$)	456 ± 105*	2.7 ± 0.3*	1.9 ± 0.2*

[a]This separate group of animals was fed LF diet or LF plus 3 d of ASA. Animals then underwent surgery and bile drainage or had livers removed without surgery. Surgical intervention did not affect the results of ASA treatment. Values are mean ± SD. * = Significantly different from comparable animals not receiving ASA, $P < 0.05$. See Tables 1 and 3 for abbreviations.

rats on FO diet. An exaggerated response in FO-fed rats has been previously reported after T3 treatment (21), and was again seen in the current study. Because of variability in responses of the six FO hypothyroid animals given T3, augmentation above the secretion rate seen in LF rats given T3 did not reach statistical significance. The bile cholesterol response to co-administration of T3 plus ASA to hypothyroid rats suggests that the two mechanisms are not identical, since augmented cholesterol secretion was synergistic in FO-fed but not in LF-fed rats.

The mechanism for the observed ASA actions on bile lipid secretion is not clear. Aspirin is an organic acid which is absorbed, deacetylated to SA, and carried by albumin in blood. Its principal excretory route is renal, but we were able to detect SA in bile. Certain organic anions cause choleresis in association with reduced cholesterol and phospholipid secretion (14,15). We found no effect of ASA on bile flow rate, as opposed to reports of choleresis without change in bile lipid secretion after intravenous infusion of ASA or SA. Thus, the oral ASA effects are not typical of organic anion effects.

Anti-inflammatory effects of aspirin might account for the results observed. Laparotomy with bile duct cannulation and drainage is a traditional method for studying bile secretion in the rat. The surgical intervention could cause cytokine-mediated cholestasis during 2 h of collection, an action prevented by ASA treatment. Since bile collection could only be measured by drainage, we could not test this thesis directly. As shown in Table 5, we found no difference in hepatic HMG-CoA reductase activity or cholesterol ester content in chow-fed rats when measured immediately at time of sacrifice or 2 h after laparotomy and bile drainage. Consistent with findings in Table 1, however, ASA pre-fed rats had higher levels of reductase activity and reduced levels of cholesterol ester content, whether operated or unoperated. Bile cholesterol secretion, of course, could not be measured in unoperated animals. Reductase activity and ester content, two measurements associated with increased bile cholesterol secretion, were unaffected by the surgical procedure. This suggests that ASA's effect was not merely to prevent a cytokine-mediated artifact of this model.

The isolated hepatocyte study found increased esterification of cell membrane [^{3}H] cholesterol in animals fed ASA, but not as a result of hepatocyte incubation with ASA during 4-h culture. These experiments, coupled with absence of ACAT change, suggest that the bile cholesterol hypersecretion did not result from impaired esterification. It is likely that bile lipid secretion differs in isolated hepatocytes compared to intact liver. The increased ester formation observed in cells isolated from ASA-treated rats might have resulted from their state of cholesterol and ester depletion (Table 1), coupled with cessation of the biliary cholesterol hypersecretion after cell isolation.

It is possible that ASA might alter hepatocyte membrane composition or function (34). Hepatic inulin clearance was not altered in the i.v. sodium SA study (18), suggesting that SA does not alter liver permeability. However, altered cellular membrane lipid transport is possibile. Thus, ASA might facilitate cholesterol (and phospholipid) transfer from one pool to another, or influence transport of lipid vesicles to the canalicular membrane (35). The similarity of ASA effect to that of T3 suggests this possibility, as do the synergistic effects observed for ASA with treatments by FO and T3—agents which also affect membrane composition.

ASA is a cyclooxygenase inhibitor. Altered prostaglandin metabolism may have been intermediary in the bile lipid hypersecretion. Since unsaturated fatty acids, such as n-3 fatty acids, are substrates for cyclooxygenase, lipoxygenases and other oxygenase enzymes, prostaglandins or other eicosanoids may be a link to explain the synergism seen in FO-fed hypothyroid animals. This possibility warrants further evaluation. It also remains to be seen whether ASA-altered bile lipid secretion is specific to the young rat or occurs in other species, including humans.

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γ-Linolenic Acid-Containing Diet Attenuates Bleomycin-Induced Lung Fibrosis in Hamsters

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ABSTRACT: Although bleomycin (BLM), an antineoplastic drug, is used in the treatment of a variety of tumors, the mechanism(s) that contribute to its induced lung injury and fibrosis are not fully elucidated. Since alterations in the levels of certain fatty acid metabolites have been associated with BLM-induced lung injury, we tested the effects of dietary γ-linolenic acid (GLA)-containing evening primrose oil on BLM-induced morphological alterations in the hamster lung, the marked elevation of tissue hydroxyproline (a marker for collagen synthesis), and elevated generation of arachidonic acid metabolites (marker of inflammatory mediators). Our data revealed that after 14 d of dietary GLA-containing oil (i) BLM-induced elevation of lung hydroxyproline was suppressed ($P < 0.05$), (ii) the marked BLM-induced elevation of lung leukotriene B_4 (LTB_4) (a marker of polymorphonuclear generation of proinflammatory LTB_4) was significantly suppressed ($P < 0.05$). The decrease in LTB_4 was accompanied by marked elevations ($P < 0.05$) of lung prostaglandin E_1 (PGE_1) and 15-hydroxyeicosatrienoic acid (15-HETrE), both with known antiinflammatory properties. Taken together, data from these studies suggest that dietary GLA-containing oil contributes to tissue elevation of PGE_1 and 15-HETrE, which *in vivo* may attenuate lung inflammation and fibrosis.
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Bleomycin (BLM), a mixture of cytotoxic glycopeptides, is used widely as an antineoplastic drug in the treatment of lymphoma, testicular and squamous cell tumors (1), since it has minimal hematopoietic toxicity or immunosuppressive activity. However, this therapy is often complicated by a dose-dependent induction of interstitial pneumonitis that progresses into interstitial pulmonary fibrosis (2), thus, limiting its long-term use. Although the pathogenic mechanisms that contribute to the development of BLM-induced lung injury and fibrosis are not fully elucidated, the search for antifibrotic agents persists in order to prevent the development of lung fibrosis.

The intratracheal instillation of BLM into rodents has been reported to induce fibrosis similar to that seen in humans (3,4); thus, these animals have formed useful models for investigating the biochemical mechanisms of the pulmonary fibrosis. Evidence of the involvement of inflammatory cells and their mediators in BLM-induced pulmonary fibrosis has been reported (5). The pathogenesis of the induced inflammation is characterized initially by edema, hemorrhage, and cellular infiltrates containing predominantly polymorphonuclear (PMN) (6,7) in the vascular, interstitial, and alveolar spaces of the lung. Since neutrophils are known to elaborate potent inflammatory eicosanoids particularly leukotriene (LTB_4), which is generated from arachidonic acid (AA), the possibility exists that eicosanoid mediators may contribute, at least in part, to the initial inflammatory response and ultimately to the pulmonary fibrosis. The reported alterations of prostaglandins and leukotrienes in certain lung disorders (8–10) suggest that these AA metabolites may be pivotal in the early pathogenic phases of lung injury. Additionally, BLM has been reported to induce the generation of reactive oxygen species (11). The increase and the availability of these reactive oxygen species have been associated with increased biosynthesis of prostaglandins (PG) (12). Also interesting, PG and related AA metabolites have been reported to modulate the growth and function of fibroblasts responsible for collagen synthesis (13,14). Taken together, the regulation of excessive generation of inflammatory eicosanoids seems pivotal in the suppression of BLM-induced lung injury. Thus, the need to evolve a therapeutic strategy with minimal side effects seems prudent.

There is a heightened interest that the dietary intake of certain polyunsaturated fatty acids (PUFA) contained in fish oil and/or in vegetable oils may function *in vivo* to alleviate inflammatory disorders. The initial excitement was that dietary PUFA of the n-3 series—α-linolenic acid (18.3n-3), eicosapentaenoic acid (EPA) (20:5n-3) and docosahexaenoic acid (DHA) (22.6 n-3)—could modulate a variety of chronic in-

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Abbreviations: AA, arachidonic acid; BLM, bleomycin; B/S, basal-saline; CO, corn oil; DHA, docosahexaenoic acid; DGLA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; 15-HETrE, 15-hydroxyeicosatrienoic acid; 13-HODE, 13-hydroxy-9,11-octadecadienoic acid; LA, linoleic acid; LTB_4, leukotriene B_4; ODS, octadecylsilyl; PC, phophatidylcholine; PE, phosphatidylethanolamine; PG, prostaglandin; PMN, polymorphonuclear; PUFA, polyunsaturated fatty acid; RP-HPLC, reverse-phase high-performance liquid chromatography; TLC, thin-layer chromatography.

flammatory diseases. These reports prompted investigations into clarifying the basis of the effects of the dietary intake of fish oil (15–18).

Similarly, the PUFA of the n-6 series, particularly γ-linolenic acid (GLA) (18:3n-6), which is a constituent of evening primrose oil and borage oil, have also generated excitement because of reports of the clinical improvement of patients with atopic eczema (a chronic and pruritic inflammatory skin disorder) after oral administration of evening primrose oil (19,20). The ingested GLA is elongated *in vivo to* dihomo-γ-linoleic acid (DGLA), a substrate for PGE_1. This prostanoid has been reported to exert a variety of effects, including antiinflammatory properties. In a recent clinical study, continuous external administration of GLA-containing oil to injured patients promoted DGLA incorporation into red cell phospholipids without increasing AA (21). The short-term continuous enteral feeding of GLA and/or EPA-containing oil attenuated endoxin-induced acute lung injury in pigs and rats (22). It has also been reported that supplementation of human diet with GLA-containing oil (borage oil) suppressed the ability of isolated normal neutrophils to generate LTB_4. Furthermore, the clinical efficacy of dietary GLA-containing oils in patients with rheumatoid arthritis has been reported (23–25).

Based on these promising findings, we reasoned that dietary GLA-containing oil (evening primrose oil) may suppress BLM-induced lung inflammation/interstitial pulmonary fibrosis. This possibility is based on the premise that dietary GLA will be elongated *in vivo* to DGLA which in turn is metabolized into two antiiflammatory metabolites: PGE_1 and 15-HETrE. To test this hypothesis, we supplemented the diet of the BLM-treated hamsters with GLA-containing evening primrose oil in order to determine its effect on: (i) lung eicosanoids and (ii) BLM-induced interstitial pulmonary fibrosis. The abnormality induced by intratracheal instillation of a single bolus of BLM to hamsters has been characterized to initiate interstitial pneumonitis which progresses to fibrosis.

MATERIALS AND METHODS

Animals. Male golden Syrian hamsters (weighing 80–100 g) were obtained from Simonsen Inc. (Gilroy, CA). The animals were housed in groups of 3–4 hamsters per cage in facilities approved by the American Association for the Accreditation of Laboratory Animal Care of the University of California, Davis. The facility provided filtered air, a constant temperature and humidity, and a 12-h/12-h light/dark cycle. The hamsters were initially maintained on Rodent Laboratory Chow 500 (Purina Mills, Inc., St. Louis, MO) with access to water *ad libidum*. The animals were allowed 1 wk to acclimatize to local conditions prior to dietary and treatment modifications.

Study design. The animals were randomly divided into four groups of 14 animals each. There were two control groups: (A) those who received basal-saline diet plus corn oil (B/S + CO) and (B) those who received basal-saline diet plus evening primrose oil (B/S + EPO). There were two experimental hamster groups: (C) those who received basal-saline diet plus BLM plus corn oil (B/S + BLM + CO), and (D) those who received basal-saline diet plus BLM plus EPO simultaneously (B/S + BLM + EPO). The composition of the basal diet is shown in Table 1. The fatty acid composition of the dietary oils is shown in Table 2.

TABLE 1
Composition of the Basal Diet

Constituents	Amount (g/100 g dry wt)
Oil[a]	10
Casein (vitamin-free)[b]	24
L-Cystine[b]	0.3
Glucose[b]	33
Corn starch[b]	20
Cellulose[c]	3
DL-Methionine	2
Mineral mix[d]	4
Vitamin mix[e]	4

[a]The oils added were CO (10% corn oil) and EPO (10% evening primrose oil). Corn oil was purchased from ICN Nutritional Biochemicals (Cleveland, OH). Evening primrose oil was a generous gift from Sid Tracy (Traco Labs, Champaign, IL).
[b]Source: ICN.
[c]Alphacel nonnutritive bulk (ICN).
[d]Custom hamster mineral mix, ICN catalog number 960200 (g/kg diet mix): $CaCO_3$, 298; $CaHPO_4 \cdot 2H_2O$, 74; $CuSO_4 \cdot 5H_2O$, 0.03; $MgSO_4 \cdot 7H_2O$, 107; $MnSO_4 \cdot H_2O$, 4.9; KI, 0.8; K_2HPO_4, 321; NaCl, 166; $ZnCl_2$, 0.5; ferric citrate, 27.3; NaF, 0.05; $CoCl_2$, 0.06; Na_2SeO_3, 0.025; $CrK(SO_4)_2 \cdot 12H_2O$, 0.5.
[e]ICN vitamin diet fortification mix provided the following (mg/kg) except as noted: vitamin A acetate, 20,000 IU/kg; vitamin D_2, 3000 IU/kg; pyridoxine-HCl, 22; α-tocopherol, 100 IU/kg; menadione, 5; biotin, 5; choline chloride, 3000; folic acid, 3; inositol, 150; niacin, 100; calcium pantothenate, 66; riboflavin, 22; thiamine-HCl, 30; vitamin B_{12}, 30; ascorbic acid, 900.

Treatment of animals. The experimental animals were placed under pentobarbital anesthesia (75–85 mg/kg) and received intratracheally one dose of BLM sulfate dissolved in saline (7.5 U/5 mL/kg body weight) as previously described (26). The control animals received the same volume of sterile isotonic saline. Fourteen days after the intratracheal administration of BLM and dietary feedings, the animals were euthanized by an overdose with phenobarbital (90–120 mg/kg) given intraperitoneally.

For biochemical determinations, the lungs were perfused *in situ* from the right side of the heart with ice-cold isotonic saline. The lung lobes were dissected free of nonparenchyma tissue, rinsed in ice-cold saline to wash off the blood, and immediately frozen in liquid nitrogen. The frozen lungs were stored at −80°C until they were processed for: tissue fatty acids in lung phospholipids, endogenous levels of lung cyclooxygenase/lipoxygenase products, and endogenous levels of tissue hydroxyproline.

Analyses of tissue lipids. The frozen lung tissue was thawed, minced, and homogenized in saline. A portion of the homogenate was acidified to pH 3.0 and then passed through ODS (C_{18}) silica columns (Sep-Pak silica cartridge; Waters

TABLE 2
Fatty Acid Composition of Dietary Oils[a]

	Fatty acid (%)	
Fatty acid	Corn oil	Evening primrose oil
16:0	10.5	6.3
16:1n-7	0.1	trace
18:0	1.8	1.8
18:1n-9	27.3	6.5
18:2n-6	60.2	75.4
18:3n-6	0.1	10.0

[a]Details of transmethylation and preparations of fatty acid methyl esters are described in the Materials and Methods section. The number after "n" indicates the number of carbon atoms from the methyl end of the acyl chain to the nearest double bond. Values are expressed as percentage of total fatty acids for corn oil (100%), evening primrose oil (100%). Only major fatty acids were computed.

Chromatography Division, Milford, MA). Briefly, a 6-mL Sep-Pak column was initially preconditioned by passage of 6 mL of methanol (MeOH), followed by 6 mL of H_2O, and then the acidified lung homogenate was applied to the preconditioned column. An initial percolation of petroleum ether onto the column eluted the neutral lipids. This fraction was not processsed further. Next, the polar phospholipids were eluted with 25% MeOH in water, and finally, the PG/leukotrienes/hydroxy fatty acids were eluted together with 100% MeOH. The fraction which contained the mixture of PG, leukotrienes, and hydroxy acids was collected and dried under a stream of N_2 gas. The residue was resuspended in a minimal amount of chloroform/MeOH (2:1, vol/vol), and aliquots were used for determination of the eicosanoids. Radioactive prostanoid was used to determine recovery from the Sep-Pak column. Recovery of ^{14}C-PGE$_2$ is approximately 80–90%.

Identification of the cyclooxygenase products. A portion of the MeOH fraction from the Sep-Pak column was transferred into a reaction vial and dried under N_2 gas. To the dry residue was added 100 µL of *p*-bromophenacyl bromide and 2 µL of *N,N*-diisopropylethylamine (27), and the mixture was incubated for 2 h at room temperature. Authentic PG standards—PGE$_2$, PGE$_{2\infty}$, PGD$_2$, and PGE$_1$—were similarly treated with the same chemical reagents as described above. After incubations, the reaction mixtures were extracted sequentially with ethyl acetate, citrate buffer, and phosphate buffer. The ethyl acetate fraction was dried under N_2 gas and the residue reconstituted in acetonitrile/H_2O (1:1, vol/vol). The resulting *p*-bromophenacyl bromide esters of the PG were separated on a C_{18}-Ultrasphere ODS, 5-µm reverse-phase high-performance liquid chromatography (RP-HPLC) column (25 cm × 4.6 µm, i.d.) (Beckman, Berkeley, CA). The products were eluted isocratically, using a mobile phase of acetonitrile/H_2O (1:1, vol/vol) at a flow rate of 2 mL/min. The absorbance of eluted compounds at 254 nm was automatically recorded using a Beckman System Gold Model 168 detector. Quantitation was determined by comparison with authentic standards.

Identification of lipoxygenase products. Another portion from the MeOH fraction (which contained lipoxygenase products) was dried under N_2 gas and then resuspended in ethanol. An aliquot was injected into the Beckman Ultrasphere 5 µm ODS (RP-HPLC) column as previously described (28). The mobile phase, consisting of MeOH/water/acetic acid (70:30:0.01, by vol) was run first at a flow rate of 0.8 mL/min from 0–10 min, and then increased to 1.8 mL/min for 10–60 min. The separated lipoxygenase products were monitored by characteristic wavelength absorbances on two channels: Channel A was monitored mainly for the leukotrienes at 270 nm (0–20 min) and for the hydroxy fatty acids at 234 nm (20–60 min). Channel B was monitored at 280 nm (0–60 min) for any novel metabolites. Quantitations were as described above, using known amounts of authentic standards.

Identification of lung phospholipid fatty acids. The polar phospholipid fraction eluted with 25% MeOH from the Sep-Pak was dried under N_2 gas and then applied to thin-layer chromatographic (TLC) plates coated with Silica Gel G (0.25-mm thickness) (Merck, Darmstadt, Germany). The plates were developed in the solvent system: chloroform/MeOH/acetic acid/water (50:37.5:3.5:2, by vol) (28). The phospholipids on the TLC plates were visualized under ultraviolet light after spraying with 0.2% 2′, 7′-dichlorofluorescein in ethanol. The TLC bands that correlated with total phospholipids were scraped and eluted with chloroform/MeOH (2:1, vol/vol). An internal standard, heptadecanoate, was added to the mixture and then dried. The fatty acids in the total phospholipids were transmethylated in 6% methanolic HCl, and the resulting fatty acid methyl esters were separated and quantitated in a Shimadzu model GC-17A gas chromatograph (Pleasanton, CA) equipped with a DB-225 fused-silica capillary column (50% cyanopropylphenyl, 0.15-mm film thickness) 30 m × 0.25 mm i.d. (J&W Scientific, Rancho Cordova, CA). Hydrogen (36 cm/s) was used as the carrier gas. The oven was run isothermally at 200°C, and detection of the fatty acid methyl esters was performed with a flame-ionization detector.

Assay for lung hydroxyproline. Hydroxyproline was assayed as previously described (29). Briefly, after thawing and homogenizing the lung tissue, 1.0 mL aliquots of the homogenate were transferred into a tube and precipitated with 0.25 mL of 50% cold trichloroacetic acid. The samples were thoroughly mixed with trichloroacetic acid, placed on ice for 10 min, and then centrifuged at 1500 × *g* for 20 min. The recovered supernatant from each sample was discarded, and each precipitate hydrolyzed overnight in 2 mL of 6 N HCl at 100°C. The concentration of hydroxyproline was measured for each sample by the method of Woessner (30).

Histopathology. Histological studies were performed as previously described (29) on 2 to 4 randomly assigned animals from each treatment group. Briefly, animals were first anesthetized and exsanguinated by transecting the descending aorta. After opening the thoracic cavity, the heart of each animal was tied at its base to isolate the pulmonary vasculature. The trachea was cannulated, and the lungs were fixed by airway instillation with a cacodylate-buffered, glutaraldehyde, paraformaldehyde fixative (440 mosm, pH 7.4) at 30 cm of H_2O pressure. Lungs were fixed for at least 2 h and then the left, right cranial, and right caudal lobes were cut in at

least two sagittal slabs. Stratified sampling provided the best estimate of total lung structure (31). Each slab was dehydrated in 95% ethanol and embedded in paraffin. Sections of each lobe were cut and stained with hematoxylin and eosin for general histopathology and sirius red, which is specific for collagen (32). A lesion was defined as thickening of intralveolar septa due to edematous swelling, the presence of interstitial inflammatory cells, fibrosis, hyperplastic epithelial cells, or airway inflammatory cells.

Statistical analysis. Data were subjected to one-way-analysis of variance. For comparison among the dietary groups [control (CO or EPO) BLM-treated/CO and BLM-treated/EPO], the software Microsoft Office (Excel 5.1) was used. The upper limit of significance chosen was $P < 0.05$. Values in the text are means + standard error (SEM). The biochemical data were expressed on a per lung basis (33) to avoid the artifactual lowering of the values in BLM-treated animals due to presence of proteins of extrapulmonary origin.

RESULTS

GLA-Containing diet attenuates BLM-induced morphological alteration of normal hamster lung. Representative photomicrographs of lungs from hamsters in: (A) control (B/S + EPO), (B) BLM-treated (B/S + BLM + CO), and (C) BLM-treated, GLA-containing EPO-fed (B/S + BLM + EPO) hamster lungs are shown in Figure 1. Lungs from control hamsters (B/S + CO) and (B/S + EPO) showed normal acini with their interalveolar septa and a lack of alveolar inflammatory cells. Lungs from B/S diet plus BLM plus CO-treated hamsters (B/S + BLM + CO) show moderate focal to severe diffuse lung lesions. Lesions of moderate severity were proximal acinar in location and were comprised of septal thickening associated with alveolar and interstitial mononuclear cell inflammation. More severe lesions showed focal fibrotic consolidation and diffuse interstitial and alveolar mononuclear cell inflammation. In contrast, lung from hamsters that received B/S diet plus BLM plus EPO (B/S + BLM + EPO) showed mild multifocal septal thickening and inflammation in proximal acini. These lesions comprised a very small percentage of this lung.

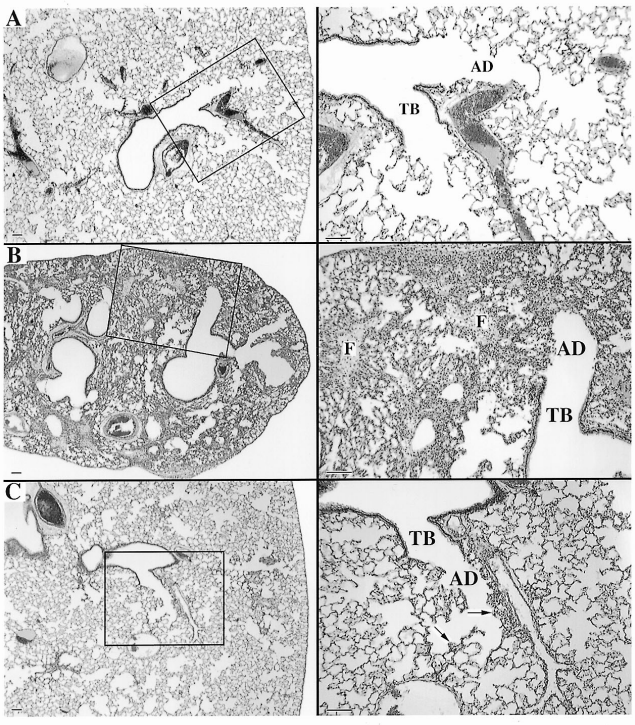

FIG. 1. Effect of γ-linolenic acid (GLA)-containing diet on altered morphologic changes in bleomycin-induced lung fibrosis. Representative photomicrographs of hamster lungs from: (A) control basal/saline (B/S) + evening primrose oil (EPO), (B) bleomycin (BLM)-treated plus corn oil (CO)-fed (B/S + BLM + CO), and (C), BLM-treated plus GLA-containing EPO-fed (B/S + BLM + EPO). The micrographs on the right are inserts (rectangles) of the lower magnification micrographs to the left. TB (representing the terminal bronchiole) and AD (representing the alveolar duct) are indicated in the magnified rectangular inserts. The bars (= 100 μm) are located horizontally on the lower left corner of the inserts. Note the normal acini in "A" with thin interalveolar septa and the absence of alveolar inflammatory cells. In contrast, in B, note the presence of fibrotic face (F) and the abundant alveolar and interstitial inflammatory cells. In C, note the mild interstitial inflammatory lesions (arrows) present in proximal acini.

*GLA-containing diet suppresses (*in vivo*) levels of (BLM)-induced lung hydroxyproline.* Lung collagen (an index of pulmonary fibrosis) was estimated by measuring tissue content of hydroxyproline. The data in Figure 2 revealed that intratracheal instillation of one dose of BLM (7.5 V/kg body weight) induced a marked increase (approximately twofold) in lung hydroxyproline after 14 d, when compared to the two control dietary groups: B/S + CO and B/S + EPO. Feeding of GLA-containing EPO-diet to the BLM-treated animals significantly suppressed (approximately 40%) the BLM-induced elevation of lung hydroxyproline. This GLA-suppressive effect is statistically significant ($P < 0.05$).

Effect of GLA-containing diet on total and individual lung phospholipid PUFA fatty acids. The distribution of PUFA in lung total and individual phospholipids after supplementation of hamster diet with EPO is shown in Table 3. The results in Table 3A revealed significant increases in endogenous DGLA (20:3n-6), the elongation product of GLA (18:3n-6), in all the lung total phospholipids of both the control animals and the experimental animals fed GLA-containing evening primrose oil. Moderate increases in AA (20:4n-6) were evident in all the animals when diets were supplemented with EPO. However, the examination of the distribution of PUFA in the two major lung phospholipids—phosphatidylcholine (PC) and phosphatidylethanolamine (PE)—shown in Table 3B and 3C revealed a striking difference in tissue AA. The data presented represent the mol% of the PUFA in PC or PE, respectively. For instance, lungs of all the animals fed EPO-supplemented diet (rich in GLA) had significantly elevated levels of DGLA in the PC when compared to PE, the level of PE being barely detectable. In contrast, the lung AA was significantly higher in PE than in PC particularly in the B/S + CO animal

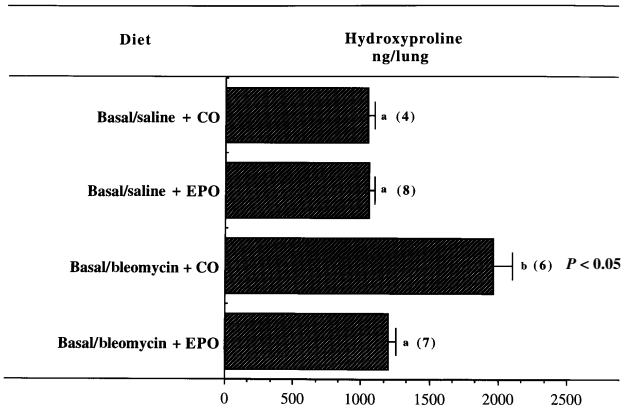

FIG. 2. GLA-containing diet suppressed BLM-induced elevation of lung hydroxyproline. Lungs were removed and homogenized in ice-cold saline from the hamsters in dietary groups: (i) B/S + CO, (ii) B/S + EPO, (iii) B/S + BLM + CO, (iv) B/S + BLM + EPO. BLM (7.5 U/6 mL/kg) was instilled intratracheally into each animal. Hydroxyproline was assayed as described in the Materials and Methods section. Values are means ± SEM. BLM-treated animals fed GLA-containing EPO (BS + BLM + EPO) significantly ($P < 0.05$) suppressed BLM-induced elevation of hydroxyproline when compared to similar animals fed CO (B/S + BLM + CO). The number of animals (*n*) in each group is shown in parentheses. Values not sharing a common superscript letter are significantly different ($P < 0.05$). See Figure 1 for abbreviations.

group. The reason for the elevated AA in PE relative to PC is presently unclear. This possibility remains to be further elucidated. It appears that these two important C_{20} carbon PUFA (DGLA and AA) are preferentially incorporated into these two major phospholipids. Furthermore, because of inadequate sample size, it was not possible to generate PUFA values for 18:2n-6 and 20:4n-6, in PE, hence, the samples from six animals were pooled and analyzed, and no SEM are shown on Table 3C.

Effect of GLA-containing diet on in vivo *generation of lung prostaglandins.* Since the lung as well as the infiltrating leukocytes are likely to generate PG in the lung, we examined the effects of GLA-supplemented diet on this profile. PGE_1, a cyclooxygenase metabolite of DGLA, was approximately sevenfold higher in the control EPO-fed animals when compared to the control CO-fed animals, which generated only moderate amounts of PGE_1, as shown in Figure 3. However, when BLM-treated animals were fed EPO, the tissue level of PGE_1 in the BLM-treated group was fivefold higher than in the CO-fed animals (Fig. 3). Thus, dietary supplementation with EPO resulted in increased lung levels of PGE_1 in both the control, EPO-fed, and the EPO-fed BLM-treated animals.

The generation of PGE_2 and $PGF_{2\infty}$, metabolites of AA, was high among all the groups of animals, as shown in Table 4, indicating a high level of AA in this tissue, presumably owing to increased release of AA by phospholipase A_2 activity. This hydrolytic activity was not investigated in this study. Interestingly, the generation of $PGF_{2\alpha}$ in EPO-fed animals was elevated when compared to normal CO-fed animals. The significance of this alteration is at present unclear.

Effect of GLA-containing diet on in vivo *generation of lung monohydroxy fatty acids.* The possibility that another potent antiinflammatory hydroxy fatty acid from dietary GLA may be generated in the EPO-fed animals prompted us to examine the lung homogenates for this 15-lipoxygenase metabolite. In control EPO-fed animals, and EPO-fed BLM-treated animals, the tissue levels of 15-hydroxyeicosatrienoic acid (15-HETrE), a metabolite of DGLA, were elevated when compared to CO-fed animals as shown in Figure 4. Interestingly, the marked increase in 15-HETrE in these two animal groups paralleled corresponding increases in 13-hydroxy-

TABLE 3
Effect of Dietary γ-Linolenic Acid-Containing Evening Primrose Oil (EPO) on Polyunsaturated Fatty Acid Distribution in Lung Total and Individual Phospholipids, Respectively[a]

Fatty acids	Basal/saline + CO	Basal/saline + EPO	Basal/bleomycin + CO	Basal/bleomycin + EPO
	A. Mol% in total phospholipids fraction			
18:2n-6	11.6 ± 0.4[a]	16.7 ± 0.8[b]	11.8 ± 0.8[a]	15.4 ± 1.4[b]
18:3n-6	0.6 ± 0.2[a]	1.3 ± 0.1[b]	0.4 ± 0.1[a]	0.8 ± 0.3[b]
20:3n-6	0.8 ± 0.1[a]	2.0 ± 0.2[b]	0.6 ± 0.1[a]	1.6 ± 0.3[b]
20:4n-6	5.7 ± 0.3[a]	7.9 ± 0.6[b]	8.2 ± 1.4[b]	8.8 ± 0.8[b]
	B. Mol% in phosphatidylcholine fraction			
18:2n-6	3.7 ± 0.1[a]	9.6 ± 1.2[b]	4.8 ± 0.3[a]	10.6 ± 0.3[b]
18:3n-6	trace[a]	0.7 ± 0.1[b]	trace[a]	0.3 ± 0.2[b]
20:3n-6	trace[a]	0.7 ± 0.1[b]	trace[a]	0.5 ± 0.1[b]
20:4n-6	0.9 ± 0.1[a]	1.9 ± 0.4[b]	1.9 ± 0.1[a]	2.2 ± 0.4[b]
	C. Mol% in phosphatidylethanolamine fraction			
18:2n-6	7.3 ± 0.5	6.6	4.5 ± 0.3	6.1
18:3n-6	trace	trace	trace	trace
20:3n-6	trace	trace	trace	trace
20:4n-6	5.5 ± 0.2	7.0	15.2 ± 2.3	4.9

[a]Values are expressed as mol% of phospholipid fatty acids in each phospholipid fraction and represent means ± SEM ($n = 6$). Values not sharing a common superscript letter are significantly different ($P < 0.05$). See footnote to Table 1 for key to diet abbreviation. "Trace" indicates trace (< 0.1) or undetectable amounts of fatty acids; CO, corn oil.

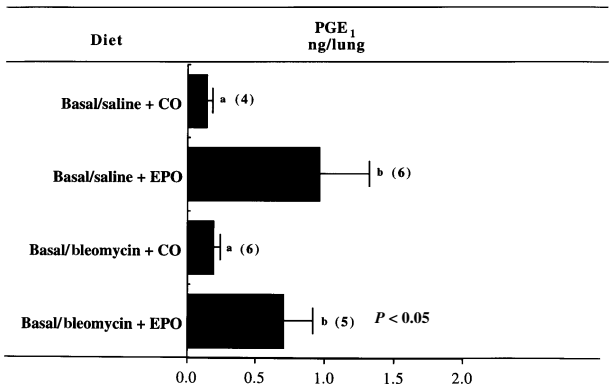

FIG. 3. Effect of dietary GLA-containing EPO on lung prostaglandins. Lungs from the animals in the four dietary groups were removed and homogenized as stated in Figure 2. The homogenates were acidified, percolated through Sep-Pak, and used for prostaglandin determination as described in the Materials and Methods section. Data represent marked generation of prostaglandin E_1 (PGE_1) in animals fed GLA-containing EPO. Results are means ± SEM from the number of animals (*n*) indicated in parentheses. See Figure 1 for other abbreviations, and Figure 2 for explanation of superscript letters.

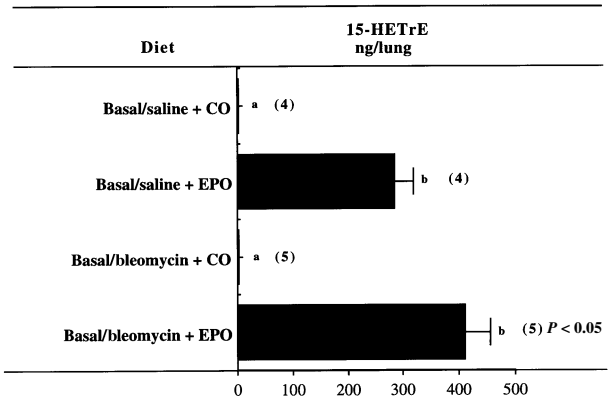

FIG. 4. Effect of dietary GLA-containing EPO on lung monohydroxy fatty acids. Lungs from the animals in the four dietary groups were removed, homogenized, and percolated through Sep-Pak as described in legend of Figure 3. Data represent marked generation of 15-hydroxyeicosatrienoic acid in animals fed GLA-containing evening primrose oil. Results are means ± SEM, with the number of animals (*n*) indicated in parentheses. See Figure 1 for other abbreviations, and Figure 2 for explanation of superscript letters.

9,11-octadecadienoic acid (13-HODE). It appears from these data that elevated tissue GLA/DGLA and/or elevated 15-HETrE may modulate the generation of 13-HODE.

Analyses of the MeOH eluent from Sep-Pak on HPLC also revealed the major hydroxy fatty acid contents from linoleic acid (LA), AA, and DGLA, respectively, as shown in Table 5. The most abundant hydroxy fatty acids in the normal CO-fed hamster lung include: 13-hydroxyoctadecadienoic acid, a metabolite of LA, and 15-HeTrE, a metabolite of AA.

GLA-containing diet suppressed in vivo *generation of BLM-induced LTB_4 in the lung.* To determine whether the intratracheal instillation of BLM into hamster lung resulted in an alteration of lung LTB_4 (a proinflammatory metabolite from AA), aliquots from the Sep-Pak column containing lipoxygenase products were subjected to fractionation on HPLC as described in the Materials and Methods section. The tissue level of LTB_4 was markedly elevated, approximately twofold in BLM-treated, CO-fed animals when compared to the two controls, as shown in Figure 5. Feeding GLA-containing EPO to the BLM-treated animals significantly ($P < 0.05$) suppressed BLM-induced lung generation of LTB_4.

DISCUSSION

Pulmonary toxicity of BLM, with the initial pneumonitis progressing to fibrosis, has been a major obstacle in the successful clinical use of this otherwise effective antineoplastic agent (2). Its wide spectrum of antitumor activity and its minimal cytotoxic effects on bone marrow justify the continued exploration of compounds that could minimize BLM-induced lung toxicity without compromising its antineoplastic efficacy.

Data from these studies revealed that supplementation of

TABLE 4
Effect of Dietary γ-Linolenic Acid-Containing EPO on Lung Prostaglandins (PG)[a]

Diets	PGE_2 (ng/mg protein)	$PGF_{2\alpha}$ (ng/mg protein)
Basal/saline + CO	317.1 ± 115.4	4.7 ± 0.4
Basal/saline + EPO	246.7 ± 58.4	16.4 ± 5.2
Basal/bleomycin + CO	202.6 ± 36.4	13.7 ± 3.1
Basal/bleomycin + EPO	203.8 ± 117.2	29.5 ± 15.2

[a]Values are expressed as means ± SEM ($n = 6$). See footnote to Table 1 for key to diet abbreviations.

TABLE 5
Effect of Dietary γ-Linolenic Acid EPO on Lung Hydroxy Acids[a]

Diets	13-HODE (ng/mg protein)	15-HETE (ng/mg protein)	12-HETE (ng/mg protein)
Basal/saline + CO	0.6 ± 0.0	0.1 ± 0.0	trace
Basal/saline + EPO	2.4 ± 0.0	0.3 ± 0.0	trace
Basal/bleomycin + CO	0.8 ± 0.1	0.2 ± 0.0	trace
Basal/bleomycin + EPO	2.7 ± 0.2	0.3 ± 0.1	trace

[a]Values are expressed as means ± SEM ($n = 6$). See footnote to Table 1 for key to diet abbreviations. "Trace" indicates trace (<0.05) or undetectable amounts; HETE, 15-hydroxyeicosatetraenoic acid; 13-HODE, 13-hydroxy-9,11-octadecadienoic acid.

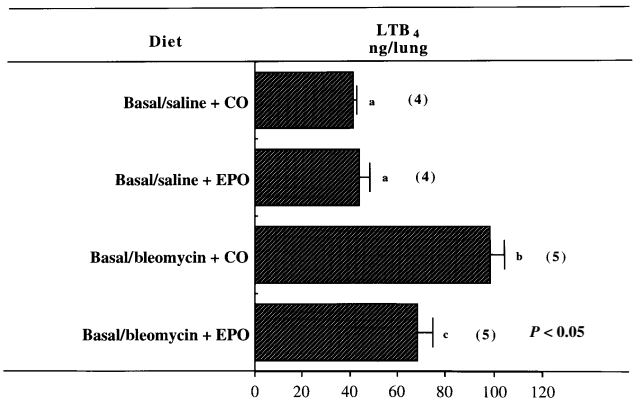

FIG. 5. GLA-containing diet suppressed *in vivo* generation of BLM-induced leukotriene B_4 in the lung. Lungs from the animals in the four dietary groups were removed, homogenized, and percolated through Sep-Pak as described in the legend for Figure 3. Animals fed GLA-containing EPO (BS + BLM + EPO) significantly ($P < 0.05$) suppressed BLM-induced generation of LTB_4 in the lung. Results are means ± SEM, with the number of animals (*n*) indicated in parentheses. See Figure 1 for other abbreviations, and Figure 2 for explanation of superscript letters.

hamster diet with EPO resulted in an elevation of DGLA (an elongation metabolite of GLA) in lung phospholipids (Table 3, A/B). This increase paralleled the elevation in the lung of two major metabolites of DGLA: (i) PGE_1 (a cyclooxygenase product of DGLA) (Fig. 3) and (ii) 15-HETrE (a 15-lipoxygenase product of DGLA) (Fig. 4). The transformation of DGLA into PGE_1 has been reported in a variety of tissues and cells (34–36). Functionally, PGE_1 has been reported to exert biological and pharmacological effects in a variety of systems. For instance, PGE_1 is linked to adenyl cyclase *via* its cell membrane receptor (37) where it increases the cyclase activity in platelets (38) and in fibroblasts (39). In fibroblasts, PGE_1 is reported to induce calcium influx through the plasma membrane (40), whereas in platelets PGE_1 inhibits intracellular calcium mobilization (41) as well as the effects of activated protein kinase C (42,43). The above reports suggest a mechanism whereby endogenous PGE_1 (presumably enhanced by its *in vivo* precursor, DGLA) may regulate function through its interactions with protein kinase C.

GLA/DGLA, as well as 15-HETrE (their 15-lipoxygenase metabolite), have been implicated in biological and pharmacological functions in a variety of systems. For instance, supplementation of guinea pig or human diet with GLA-containing oils resulted in marked elevation of DGLA in the tissue. Both guinea pig and human epidermis contains a very active elongase that catalyzes the conversion of precursor GLA into DGLA (44). *In vitro*, the DGLA is metabolized by epidermal cyclooxygenase into PGE_1, and by epidermal 15-lipoxygenase into 15-HETrE (45). *In vivo*, when guinea pig diet was supplemented with GLA-containing oil, 15-HETrE was generated in the epidermis (46). Additionally, when human diet was supplemented with GLA-containing capsules, a significant amount of DGLA was found in the phospholipids of the PMN. The elevation of DGLA in the phospholipids paralleled the decreased capacity of isolated PMN to biosynthesize LTB_4 *in vitro* (47). These observations are consistent with the *in vitro* inhibition of AA transformation into LTB_4 by 15-HETrE (45,48,49).

Taken together, GLA/DGLA, including their major cyclooxygenase metabolite (PGE_1) and its 15-lipoxygenase metabolite (15-HETrE), are potent modulators of cellular functions. Thus, it is reasonable to expect that the dietary intake of GLA-containing oil could generate both metabolites *in vivo*, which in turn can modulate the pathophysiological conditions of BLM-induced interstitial pulmonary fibrosis. Data derived from these studies are consistent with the stated speculations. The animals whose diets were supplemented with GLA-containing EPO generated elevated levels of PGE_1 and 15-HETrE when compared to control animals whose diet was supplemented with CO. Interestingly, the generation of these metabolites in BLM-treated animals correlated with the suppression of LTB_4 (a proinflammatory metabolite generated from AA). In a recent report, the constitutive activation of 5-lipoxygenase (the enzyme that catalyzes the generation of LTB_4 and peptido LTC_4 from AA) was demonstrated in the lungs of patients with idiopathic pulmonary fibrosis (50). Lung homogenates from the idiopathic pulmonary fibrosis patients contained 15-fold more LTB_4 than homogenates from nonfibrotic lungs. Our data are consistent with the elevation of LTB_4 in this report. Our data further revealed the suppression of lung hydroxyproline (a marker for collagen accumulation) after the dietary intake of GLA-containing EPO. Interestingly, leukotrienes have been reported to exert direct effects on fibroblasts and mesenchymal cells, where they stimulate fibroblast proliferation (51) and collagen synthesis (52). These *in vivo* possibilities imply that the dietary intake of highly purified GLA-containing oil or sufficient intake of constituent PUFA of these oils may serve as a fitting adjunct with negligible side effects for alleviating BLM-induced lung inflammation and fibrosis.

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Oleanolic Acid and Ursolic Acid Stabilize Liposomal Membranes

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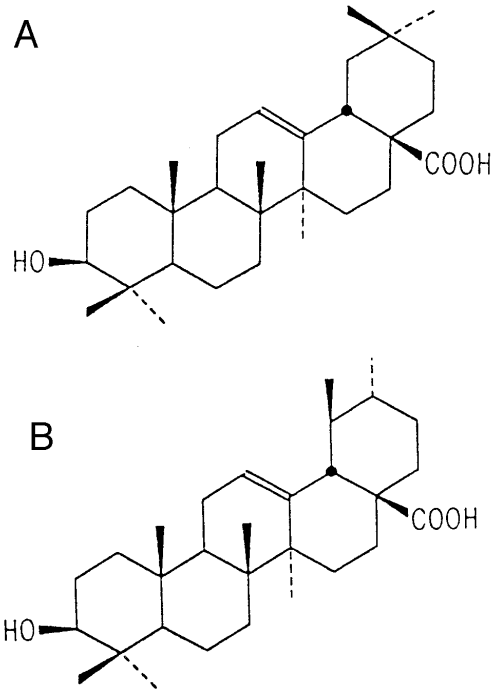
ABSTRACT: The effects of oleanolic acid (OA) and ursolic acid (UA) on the fluidity and stability of dipalmitoyl phosphatidylcholine (DPPC) liposomal membrane were monitored by measuring the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene labeled in the liposomal membrane and the leakage of calcein from the probe-encapsulated liposomes. The experiments with the liposomes made of DPPC and OA or UA showed that OA and UA exhibited a moderate fluidity-modulating effect for the liquid-crystalline liposomal membrane, and a strong condensing effect for both crystalline and liquid-crystalline liposomal membranes. Their effects were comparable to those of cholesterol. These results suggest that their fluidity-modulating and condensing effects might have some implications in their biological functions.

Lipids 32, 769–773 (1997).

Oleanolic acid (OA) (Scheme 1A) and ursolic acid (UA) (Scheme 1B) are pentacyclic triterpene acids which are widely distributed in plants, and their derivatives are aglycones of many naturally occurring saponins (1). UA is also the main component of protective wax-like coatings of many fruits (2). Pentacyclic triterpene acids may possibly enhance the mechanical barrier functions of cell membranes in plants.

Cholesterol (Cho) is well known to enhance the mechanical barrier function of cell membranes in animals (3,4). Plant sterols are also expected to play such a role in plants. The function of Cho in biological membranes is understood on the basis of the studies on its effects on phospholipid liposomal membranes (5–7). In the crystalline state of the phospholipids below the phase transition temperature (T_m), Cho tends to increase the disorder of phospholipid molecules, particularly in the hydrocarbon chain (fluidizing effect), and in the liquid-crystalline state above T_m, cholesterol tightly assembles the molecules of phospholipids in bilayers and the membrane is less fluid and permeable to small hydrophilic molecules and ions (condensing effect) (8,9). In brief, cholesterol has fluidity-modulating and condensing effects for cell membranes. These properties of Cho are inherently related to its stucture:



SCHEME 1

a multicyclic, rigid planar structure, a hydrophilic 3β-hydroxyl group, and a hydrophobic side chain. It is worthwhile to note that OA and UA also have a multicyclic, rigid planar structure and a 3β-hydroxyl group, and are expected to have similar properties as Cho.

Recently, it has been reported that OA and UA have interesting biological activities such as antiangiogenetic activity (10) and cancer cell-differentiating activity (11). It has also been indicated that many cells have an absolute growth requirement for exogeneous sterol, and there exists an important link between sterol and cell growth (3). It has also been suggested that changes in growth behavior and differentiation of a variety of cell types are associated with alterations in plasma membrane fluidity (12,13). In consequence, studies on the effects of OA and UA on the fluidity and stabilization of biological membranes would be necessary for understanding the mechanisms of many biological activities of OA and UA.

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Abbreviations: Cho, cholesterol; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoyl phosphatidylcholine; OA, oleanolic acid; UA, ursolic acid.

In this report, we describe the effects of OA and UA on the fluidity and stabilization of liposomes composed of dipalmitoyl phosphatidylcholine (DPPC) by measuring the fluorescence polarization of the 1,6-diphenyl-1,3,5-hexatriene (DPH) (7) labeled in the liposomal membrane and the release of calcein entrapped in liposomes (14). Their effects were compared with those of Cho.

MATERIALS AND METHODS

Chemicals. OA, UA, DPPC, DPH, calcein, and Cho were purchased from Sigma Chemical Co. (St. Louis, MO), and used without further purification. All other reagents were of reagent grade.

Preparation of liposomes. The mixture of DPPC and various amounts of OA, UA, or Cho was dissolved in 2 mL of $CHCl_3$ and reduced to dryness by rotary evaporation, forming a film on the inside of a round-bottomed flask. The small unilamellar liposomes were prepared by suspending the film in 5 mL of 10 mM Tris buffer (pH 7.4) and sonicated for 30 min at 50°C above the T_m of DPPC.

The incorporation of DPH into the liposomal membrane was carried out by adding an aliquot of 1 mM stock solution of DPH in DMSO to make it 2×10^{-6} mM. The mixture was vigorously vortexed and employed for measurements of the fluorescence polarization of DPH. The final concentration of DPPC in liposomes was adjusted to 0.33 mM.

For the preparation of calcein-entrapped liposomes, 5 mL of 10 mM Tris buffer (pH 7.4) containing 50 mM calcein was added to the lipid film, and the mixture was sonicated as described previously. Calcein-entrapped liposomes were separated from free calcein by passing through a Sephadex G-50 column with Tris buffer as the eluent at 10°C. The concentration of DPPC was determined by Stewart method (7) and adjusted to 30 μM by the buffer.

Measurement of the membrane fluidity. The sample was excited by vertically polarized light (358 nm), and intensities of emission (430 nm) were measured through polarizers oriented parallel to the plane of excitation (*Ih*) and perpendicular to the plane of excitation (*Iv*). The fluorescence polarization value (*P*) of DPH labeled in the liposomal membranes was calculated by Equation 1,

$$P = (Ih - Iv \times \mathrm{G})/(Ih + Iv \times \mathrm{G}) \quad [1]$$

where the correction factor *G* was *Ih*/*Iv*. The *P* value was employed as a parameter of fluidity of the membrane.

Measurement of liposomal stability. Calcein is a water-soluble, self-quenching fluorescent compound, and the measurement of fluorescing calcein released from liposomes entrapping high concentrations of this compound was employed for the indication of the leakage of the content of the liposomes (14).

Keeping the calcein-entrapped liposomes at 25 or 42°C with circulating water bath, the fluorescence (*F*) was measured at excitation and emission wavelengths of 482 and 512 nm, respectively. The percentage of the calcein leakage from liposomes was calculated by Equation 2,

$$\text{calcein leakage [\%]} = 100 \times (F - F_o)/(F_t - F_o) \quad [2]$$

where F is the fluorescence intensity at time t, F_o the initial fluorescence intensity, and F_t denotes the fluorescence intensity when the liposomes were completely disrupted by addition of 10% Triton X-100 solution.

The fluorescence measurements were carried out with a Perkin-Elmer luminescence spectrophotometer model LS-5 (Norwalk, CT), provided with a thermostatic cell holder. The samples were degassed by nitrogen before the measurement.

RESULTS AND DISCUSSION

The results of the measurements of the fluorescence polarization values of the DPH labeled in the liposomal membranes made of the lipid mixture of DPPC and OA, UA, or Cho are shown in Figure 1. They show that the values decreased gradually as the temperature increased and decreased abruptly near the T_m of the liposomal membrane from crystalline to liquid-crystalline state as usual cases. The midpoint of the abrupt changes which were shown as sigmoidal curves was taken as T_m. It was 40°C for DPPC liposomes, which was slightly lower than the referred value, 41°C (7).

The addition of the additives to DPPC did not affect the *P* values at 10 mol% content of the additives or slightly lower the values at higher content of the additives at temperatures below T_m. Their differences from the control were not significant. In crystal phase, Cho has been known to increase chain flexibility by inhibiting good packing of the fatty acyl chains. However, the results of the experiments showed that the fluidizing effects of OA, UA, and Cho on the DPPC liposomal membrane in crystalline state are insignificant or not great enough to be detected by this method. On the other hand, it was clearly shown that the addition of the additives to DPPC significantly increased the *P* values in the liquid-crystalline state, and these effects are highly dose-dependent. This means that the addition of OA, UA, or Cho to the DPPC decreases the fluidity of the liposomal membrane in proportion to its concentration in liquid-crystalline state. No significant difference between the activities of OA and UA was observed. This is understandable considering the extreme similarity of structures of these two compounds. However, they were less effective than Cho.

The presence of the rigid multicyclic frame of OA, UA, and Cho restricts the flexing motion of neighboring fatty acyl chain region in the liquid-crystalline phase and reduces the fluidity. OA and UA were less effective in decreasing the fluidity than Cho at the 20 mol% or more content level, although OA and UA have a bulkier multicyclic planar structure than Cho. This suggests that not only the multicyclic planar structure of these additives but also the hydrophobic tail of Cho should be important moieties in inducing fluidity-modulating effects of these compounds on the liposomal membranes. The hydrophobic tail of Cho might be fitted better into the lipid

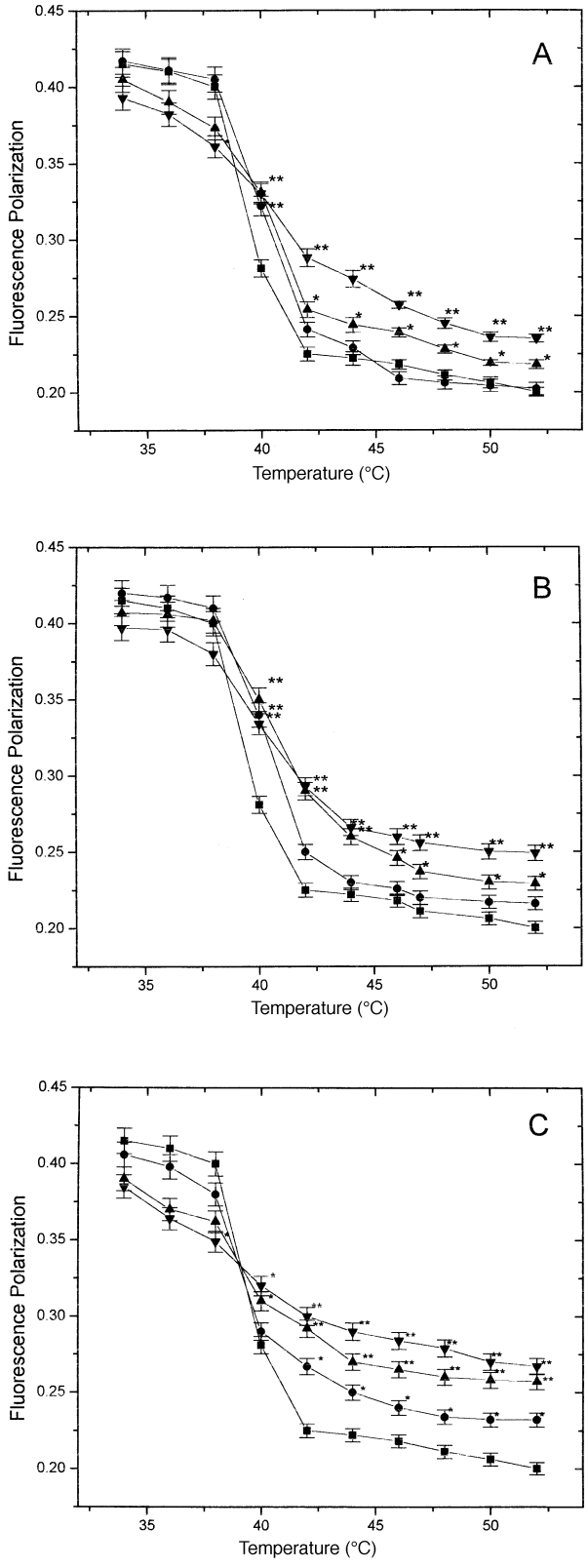


FIG. 1. Fluorescence polarization value of 1,6-diphenyl-1,3,5-hexatriene labeled in the dipalmitoyl phosphatidylcholine (DPPC)-additive liposomal membrane. Each point represents the mean of five replicate measurements ± SE. ■ DPPC, ● DPPC and 10 mol% additive, ▲ DPPC and 20 mol% additive, ▼ DPPC and 30 mol% additive. A: OA, B: UA, C: Cho. *$P < 0.05$ vs. control. ** $P < 0.01$ vs. control.

bilayer, which eventually induces more effective fluidity modulation than OA and UA.

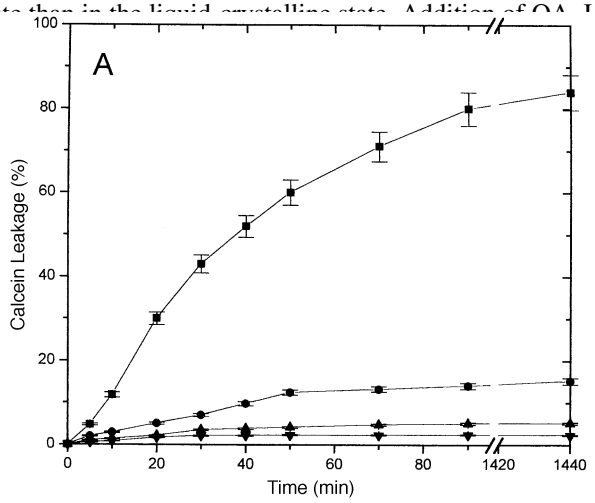
The T_m of the DPPC liposomes did not shift significantly by addition of the additives. However, the temperature range of the phase transition became broader on addition of the additives. This means that the incorporation of the additives into the lipid bilayer of the liposomal membrane reduces the size of the cooperative unit of the phase transition and induces the initiation of the phase transition at lower temperature and the completion of the transition at higher temperature. It has been reported that the presence of more than 30 mol% Cho in DPPC liposomes abolished completely the phase transition peak on the differential scanning thermogram (4). This was ascertained in this experiment. As the content of Cho in the lipid reached 20 mol%, the transition became less evident, and in the presence of 30 mol% Cho, the P value decreased monotonously with increasing temperature, and the T_m was not observed. However, even in the presence of 30 mol% OA or UA in the lipid, the phase transitions were clearly observed.

The results of the measurements of the leakage of calcein at 25 and 42°C from the liposomes made of DPPC and the additives are shown in Figures 2 and 3. They show that OA, UA, and Cho have strong membrane-stabilizing effects for the liposomes. While the liposomes made of DPPC alone were quite unstable, releasing nearly 80% of calcein from the liposomes on standing for 1 h at 25°C, the addition of OA, UA, or Cho to DPPC drastically decreased the leakage of calcein. However significant differences among the effects of these three compounds were not observed within the time period examined in this experiment at 25°C. Their membrane-stabilizing activities were also dose-dependent; the addition of 10 mol% OA, UA, or Cho considerably decreased the leakage and 20 mol% or more OA, UA, or Cho decreased the leakage of calcein nearly down to less than 3% on standing for 2 h at 25°C. These states persisted longer than 24 h.

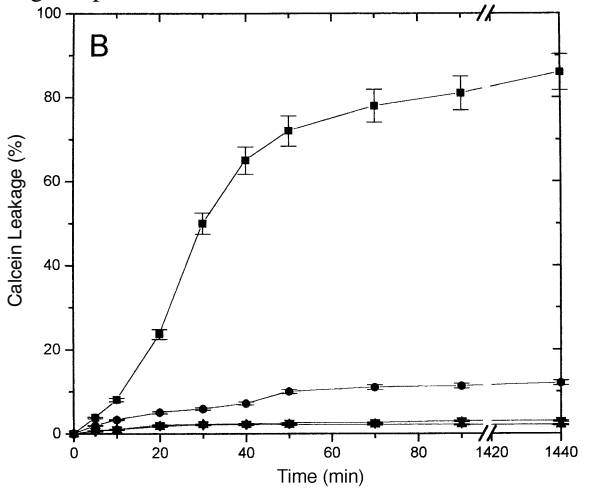
The liposomes prepared by sonication in this experiment were prestressed fragile vesicles and had a strong tendency to fuse or break. There also existed an osmotic imbalance between the internal and external solutions of the vesicles, and then water would flow down its chemical potential gradient, causing the vesicles to swell and eventually break, so leaking their contents. Although the T_m of the DPPC liposomes did not shift significantly by addition of Cho, OA or UA, the phase transition pattern was greatly perturbed by the presence of these additives. Consequently, some relation of the membrane-stabilizing effects of these additives with their effects on the phase transition could not be excluded.

The experiments on the leakage of calcein at 42°C showed that the liposomes made of DPPC alone showed approximately 70% leakage of calcein on standing for 1 h at 42°C. More leakage at 25°C than at 42°C indicates that the lipid bilayer in liquid-crystalline state is less permeable than the lipid bilayer in crystalline state in spite of the more vigorous motion of molecules and the liquid-crystalline state of the lipid at higher temperature. This indicates that there exist more defects or vacancies in the liposomal bilayer in the crystalline

state than in the liquid-crystalline state. Addition of OA, UA



sudden increase in the leakage of calcein was observed in the leakage experiments at 42°C. This should be the time re-



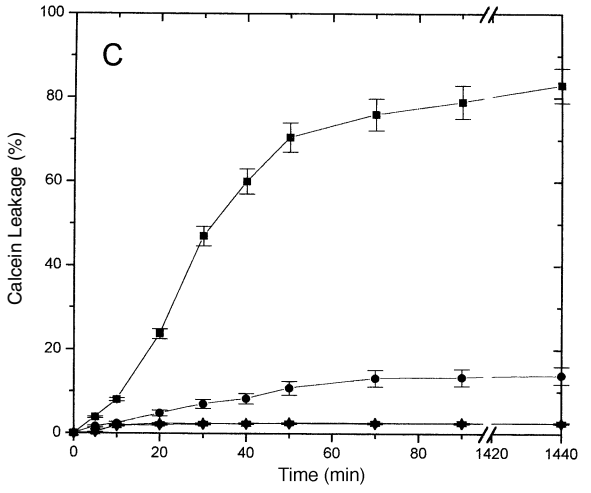
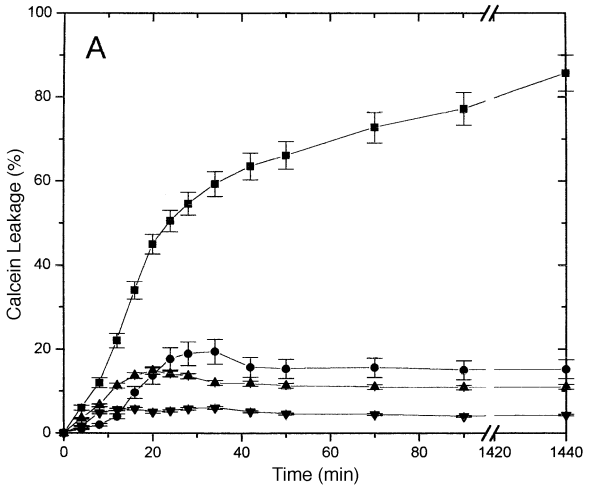
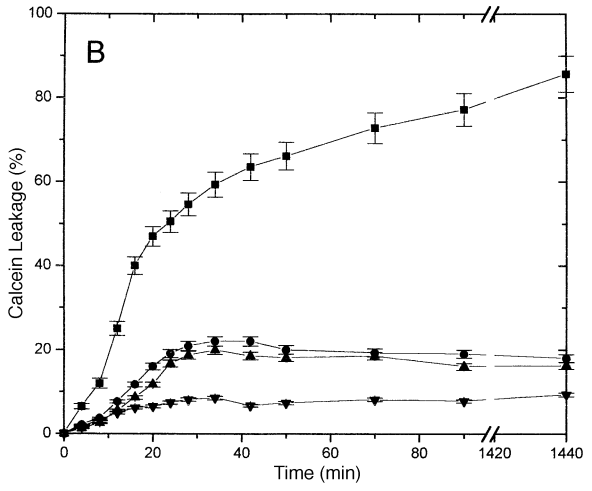


FIG. 2. Time course of the calcein leakages from the DPPC-additive liposomes at 25°C. Each point represents the mean of four replicate measurements ± SE. All datum points were significantly different ($P < 0.01$) vs. the control. ■ DPPC, ● DPPC and 10 mol% additive, ▲ DPPC and 20 mol% additive, ▼ DPPC and 30 mol% additive. A: OA, B: UA, C: Cho. See Figure 1 for abbreviation.



meability of the solute through the bilayer. The initial sudden



the absence of the hydrophobic tail in OA and UA.

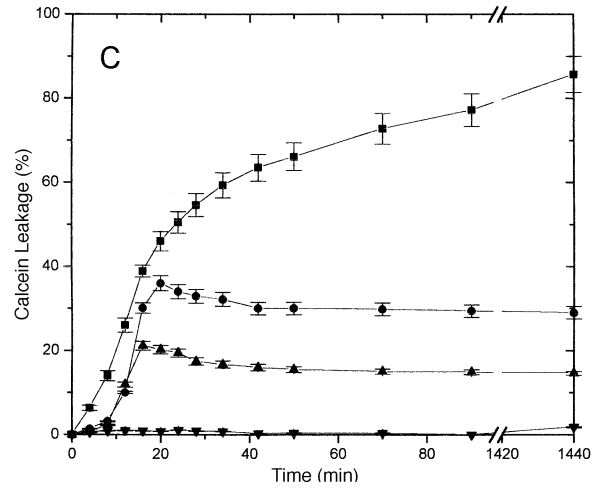


FIG. 3. Time course of the calcein leakages from the DPPC-additive liposomes at 42°C. Each point represents the mean of four replicate measurements ± SE. All datum points were significantly different ($P < 0.01$) vs. the control. ■ DPPC, ● DPPC and 10 mol% additive, ▲ DPPC and 20 mol% additive, ▼ DPPC and 30 mol% additive. A: OA, B: UA. C: Cho. See Figure 1 for abbreviation.

Cho has been well known to have a condensing effect in liquid-crystalline state of the lipids. The results of this study show that OA, UA, and Cho have a strong condensing effect for the liposomal membrane not only in the liquid-crystalline state but also in the crystalline state. The results also show that although Cho was more effective in decreasing the fluidity of liquid-crystalline liposomal membrane than OA and UA, the latter two compounds showed membrane-stabilizing effects comparable to or more than the former. This means that the fluidity modulation is not the determining factor in condensing and stabilizing the liposomal membranes. Possibly, the proper orientation of the palisade molecule in the lipid bilayers might be important for fluidity modulation, while the random distribution of multicycle planar molecules is for the condensing effect.

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An Improved Synthesis of Taurine- and Glycine-Conjugated Bile Acids

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ABSTRACT: A simple and efficient method for the synthesis of taurine- and glycine-conjugated bile acids is described. The condensation reaction was achieved by the simple mixing of unconjugated bile acid (1.0 eq.), taurine (2.0 eq.) (or glycinate ester), diethyl phosphorocyanidate (1.2 eq.) in the presence of triethylamine at room temperature for 30–60 min. Sample clean-up was effected by the use of a prepacked Sep-Pak C_{18} cartridge for reversed-phase solid extraction or by direct recrystallization, yielding the desired taurine and glycine conjugates in 89–93 and 92–96% isolated yields, respectively.
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Bile acids, synthesized from cholesterol in the liver, exist in biological fluids both in the free form at the C24 carboxylic group and, primarily, as their *N*-acyl conjugated forms with taurine or glycine (1).

In continuation of a program of synthesis of potential bile acid metabolites, a need for a supply of taurine and glycine conjugates of various bile acids as authentic reference standards prompted us to examine literature preparations of the amidated bile acids, of which there are several. Those include azide (2), mixed carbonic-carboxylic acid anhydride (2–5), activated ester (6), and carbodiimide (7) methods in peptide syntheses. In particular, the mixed carbonic-carboxylic acid anhydride method introduced by Lack *et al.* (3) and then improved by Tserng *et al.* (4), using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) as a coupling reagent, has now been widely employed for preparing amidated bile acids (8,9), because of its good yield. However, the EEDQ method still has several drawbacks and problems: it is laborious, time-consuming, requires drastic reaction conditions and complicated sample workups. These aspects are undesirable, particularly for small-scale preparation of amidated bile acids.

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Abbreviations: CA, cholic (3α,7α,12α-trihydroxy-5β-cholan-24-oic) acid; CDCA, chenodeoxycholic (3α,7α-dihydroxy-5β-cholan-24-oic) acid; DCA, deoxycholic (3α,12α-dihydroxy-5β-cholan-24-oic) acid; DEPC, diethyl phosphorocyanidate; DMF, dimethylformamide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; GCA, glycocholic acid; IR, infrared; LCA, lithocholic (3α-hydroxy-5β-cholan-24-oic) acid; ^{1}H NMR, proton nuclear magnetic resonance; TLC, thin-layer chromatography; UDCA, ursodeoxycholic (3α,7β-dihydroxy-5β-cholan-24-oic) acid.

Recently, Tohma *et al.* (10) have reported the use of diethyl phosphorocyanidate (DEPC) as an alternative coupling reagent for preparing some amidated bile acids. The method seems to be useful, but a limited number of bile acids were examined, and detailed reaction conditions and workup procedures remain unreported. We now present an improved method for the preparation of taurine- and glycine-conjugated bile acids (Table 1), using DEPC as a coupling reagent, which proceeds rapidly under mild conditions and affords a pure amidated product isolated in a good yield without requiring complicated workup and tedious column chromatographic purification steps, in contrast to previous procedures.

EXPERIMENTAL PROCEDURES

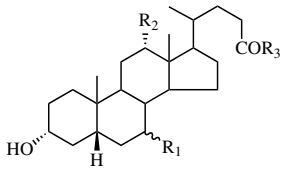
Materials and reagents. Each m.p. was determined on an electric micro hot stage and are uncorrected. Infrared (IR) spectra were obtained on a Perkin-Elmer 1600 Series Fourier transform IR spectrometer (Norwalk, CT) as KBr pellets. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Jeol FX-90Q instrument (Tokyo, Japan) at 90 MHz, with CD_3OD containing 0.1% Me_4Si as the solvent; chemical shifts are expressed in δ (ppm) relative to Me_4Si.

Lithocholic (LCA), deoxycholic (DCA), and cholic (CA) acids were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chenodeoxycholic (CDCA) and ursodeoxycholic (UDCA) acids were kindly donated by Tokyo Tanabe Co. (Tokyo, Japan). Peptide coupling reagent, DEPC, was supplied by Wako Pure Industries Ltd., and glycine methyl ester hydrochloride, taurine, and triethylamine (Et_3N) were supplied by Nacalai Tesque, Inc. (Kyoto, Japan). All other reagents and solvents were of analytical reagent grade.

Prepacked octadecyl-bonded (C_{18}) silica cartridges for reversed-phase solid extraction, Sep-Pak plus tC_{18} (sorbent weight, 400 mg) and Sep-Pak Vac tC_{18} (sorbent weight, 5 g) were from Waters Associates (Milford, MA). They were washed with 4 mL (or 50 mL) MeOH and 8 mL (or 100 mL) distilled water prior to use.

Thin-layer chromatography (TLC). Analytical TLC was performed on precoated silica gel $60F_{254}$ plates (20 cm × 20 cm, 0.25-mm layer thickness; Merck, Darmstadt, Germany). For the developing solvents, *n*-propyl alcohol/CH_3COOH/water (100:7:6, by vol) and EtOAc/*n*-hexane/CH_3COOH (50:50:1,

TABLE 1
Structures of Unconjugated and Taurine- and Glycine-Conjugated Bile Acids



		R_3		
R_1	R_2	OH	$NHCH_2CH_2SO_3Na$[a]	$NHCH_2COOH$[a]
H	H	LCA	TLCA (92)	GLCA (89)
α-OH	H	CDCA	TCDCA (93)	GCDCA[b] (92)
β-OH	H	UDCA	TUDCA (95)	GUDCA (90)
H	OH	DCA	TDCA (96)	GDCA (93)
α-OH	OH	CA	TCA (92)	GCA (93)

[a]Values in parentheses refer to isolated yields (%); taurine and glycine conjugates were prepared by methods A and B, respectively, as described in the Experimental Procedures section.
[b]Obtained as a potassium salt. Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid; LCA, lithocholic acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

by vol) mixtures were employed for taurine and glycine conjugates, respectively. The spots were detected with 10% phosphomolybdic acid in methanol and heating at 110°C.

Gas–liquid chromatography. A Shimadzu GC-14A gas chromatograph equipped with a flame-ionization detector (Tokyo, Japan) was used. It was fitted with an Ultra ALLOY-1 (HT) (Koriyama, Japan) stainless-steel capillary column (15 m × 0.25 mm i.d.) coated with a film (0.25 μm) of chemically bonded and crosslinked dimethylsiloxane and operated under the following conditions: carrier gas (helium) flow rate, 0.7 mL/min; helium linear velocity, 15.9 m/min; splitting ratio, 50:1; injector temperature, 330°C; detector temperature, 350°C; column temperature, 320°C (isothermal). Simultaneous analysis of unconjugated and glycine-conjugated bile acids was carried out as their methyl ester-trimethylsilyl ether derivatives as described previously (11).

Recovery and reproducibility tests by solid-phase extraction. Sample solutions for recovery and reproducibility tests by Sep-Pak C_{18} cartridges were prepared as follows: Known amounts (50, 70, and 80 mg) of glycocholic acid (GCA) were dissolved in phosphate buffer (pH 7.5) and adjusted to 1% solution. Each solution was applied to a preconditioned Sep-Pak plus tC_{18} cartridge and washed with 2 mL of water. GCA was then eluted with 2 mL of EtOH. In each of three experiments, approximately equal amounts (38, 38, and 39 mg; mean, 38 mg) of GCA were recovered.

General procedure for the synthesis of taurine-conjugated bile acid sodium salts (method A). To a magnetically stirred solution of an unconjugated bile acid (0.25 mmol) in dry dimethylformamide (DMF; 1 mL) were added successively powdered taurine (0.5 mmol), DEPC (0.3 mmol), and anhydrous Et_3N (0.4 mL), and the resulting suspension was stirred at room temperature for 60 min (the reaction was monitored by TLC). The reaction mixture was adjusted to pH 12–14 with 1 M NaOH and then to pH 7–8 with 10% HCl. The solution was diluted with water (9 mL), passed through a preconditioned Sep-PakVac tC_{18} cartridge, and eluted successively with water (20 mL), 25% EtOH (20 mL), and EtOH (25 mL). The last fraction, which contains the desired taurine conjugate sodium salt was evaporated to dryness under a nitrogen stream, and the residue was recrystallized from an appropriate solvent.

General procedure for the synthesis of glycine-conjugated bile acids (method B). To a magnetically stirred solution of an unconjugated bile acid (0.25 mmol) in dry DMF (1 mL) were added successively glycine methyl ester hydrochloride (0.5 mmol), DEPC (0.3 mmol), and Et_3N (0.4 mL), and the resulting suspension was stirred at room temperature for 30 min. The reaction product was extracted with EtOAc, and the combined extracts were washed with water, dried with Drierite, and evaporated to dryness. The residue was then refluxed for 30 min in 5% methanolic KOH (5 mL). Most of the solvent was evaporated under reduced pressure, and the hydrolysis product was dissolved in water (5 mL) and acidified with 5% H_2SO_4 with stirring. The precipitated solid was filtered, washed with water, and dried to give the crude glycine conjugate, which was recrystallized from an appropriate solvent. Otherwise, the KOH hydrolysis product was subjected directly to a Sep-Pak C_{18} cartridge as described above, and the desired glycine conjugate was obtained as a potassium salt.

Taurolithocholic acid sodium salt. Obtained from LCA in 89% yield; m.p., 204–208°C (MeOH-Et_2O) [(lit. m.p., 199–201°C (4)]. IR ν_{max} cm^{-1}: 3304 (OH), 1652 (C=O), 1558 (CONH), 1217, 1046 (SO_3). ^1H NMR δ:0.66 (3H, *s*, 18-Me), 0.93 (3H, *s*, 19-Me), 0.93 (3H, *d*, *J* = 5.4 Hz, 21-Me), 2.96 (2H, *t*, *J* = 5.4 Hz, $CH_2CH_2SO_3Na$), 3.62 (2H, *t*, *J* = 6.3 Hz, $CH_2CH_2SO_3Na$).

Taurodeoxycholic acid sodium salt. Obtained from DCA in 93% yield; m.p., 169–173°C (EtOAc-aq. MeOH) [(lit. m.p., 172–173°C (4)]. IR ν_{max} cm^{-1}: 3406 (OH), 1650 (C=O), 1549 (CONH), 1214, 1043 (SO_3). ^1H NMR δ: 0.69 (3H, *s*, 18-Me), 0.92 (3H, *s*, 19-Me), 0.93 (3H, *d*, *J* = 5.4 Hz, 21-Me), 2.98 (2H, *t*, *J* = 5.4 Hz, $CH_2CH_2SO_3Na$), 3.61 (2H, *t*, *J* = 6.3 Hz, $CH_2CH_2SO_3Na$).

Taurochenodeoxycholic acid sodium salt. Obtained from CDCA in 92% yield; m.p., 177–179°C (EtOH-EtOAc) [(lit. m.p., 182–184°C (4)]. IR ν_{max} cm^{-1}: 3412 (OH), 1654 (C=O), 1551 (CONH), 1221, 1052 (SO_3). ^1H NMR δ: 0.66 (3H, *s*, 18-Me), 0.91 (3H, *s*, 19-Me), 0.94 (3H, *d*, *J* = 4.5 Hz, 21-Me), 2.99 (2H, *t*, *J* = 5.4 Hz, $CH_2CH_2SO_3Na$), 3.60 (2H, *t*, *J* = 6.3Hz, $CH_2CH_2SO_3Na$).

Tauroursodeoxycholic acid sodium salt. Obtained from UDCA in 90% yield; m.p., 201–204°C (EtOH-EtOAc) [(lit. m.p., 200–204°C (4)]. IR ν_{max} cm^{-1}: 3387 (OH), 1654 (C=O), 1550 (CONH), 1223, 1054 (SO_3). ^1H NMR δ: 0.68 (3H, *s*, 18-Me), 0.95 (3H, *s*, 19-Me), 0.94 (3H, *d*, *J* = 5.4 Hz, 21-Me), 2.99 (2H, *t*, *J* = 5.4 Hz, $CH_2CH_2SO_3Na$), 3.61 (2H, *t*, *J* = 6.3 Hz, $CH_2CH_2SO_3Na$).

Taurocholic acid sodium salt. Obtained from CA in 93% yield; m.p., 181–184°C (EtOH-EtOAc) [(lit. m.p., 182–184°C (4)]. IR ν_{max} cm^{-1}: 3416 (OH), 1649 (C=O), 1549 (CONH), 1197, 1042 (SO_3). ^1H NMR δ: 0.70 (3H, *s*, 18-Me), 0.91 (3H, *s*, 19-Me), 1.01 (3H, *d*, *J* = 5.4 Hz, 21-Me), 2.98 (2H, *t*, *J* = 6.3 Hz, $CH_2CH_2SO_3Na$), 3.59 (2H, *t*, *J* = 7.2 Hz, $CH_2CH_2SO_3Na$).

Glycolithocholic acid. Obtained from LCA in 92% yield; m.p., 183–185°C (aq. EtOH) [lit. m.p., 184–186°C (1)]. IR ν_{max} cm^{-1}: 3285 (OH), 1611, 1735 (C=O), 1568 (CONH). ^1H NMR δ: 0.64 (3H, *s*, 18-Me), 0.91 (3H, *s*, 19-Me), 0.92 (3H, *d*, J = 5.4 Hz, 21-Me), 2.59 (1H, *brm*, 3 β-H), 3.97 (2H, *d*, *J* = 4.5 Hz, $NHCH_2COOH$), 6.55 (1H, *t*, *J* = 4.5 Hz, $NHCH_2COOH$).

Glycodeoxycholic acid (GDCA). Obtained from DCA in 96% yield; m.p., 191–193°C (aq. EtOH) [lit. m.p., 191–192°C (4)]. IR ν_{max} cm^{-1}: 3348 (OH), 1631, 1722 (C=O), 1531 (CONH). ^1H NMR δ: 0.67 (3H, *s*, 18-Me), 0.90 (3H, *s*, 19-Me), 0.99 (3H, *d*, *J* = 5.4 Hz, 21-Me), 3.97 (2H, *d*, *J* = 4.5 Hz, $NHCH_2COOH$), 6.68 (1H, *t*, *J* = 4.5 Hz, $NHCH_2COOH$).

Glycochenodeoxycholic acid (GCDCA) potassium salt. Obtained as the potassium salt from CDCA in 93% yield; m.p., 155–163°C (MeOH-Et_2O). IR ν_{max} cm^{-1}: 3412 (OH), 1655, 1733 (C=O), 1545 (CONH). ^1H NMR δ: 0.66 (3H, *s*, 18-Me), 0.90 (3H, *s*, 19-Me), 0.93 (3H, *d*, *J* = 5.4 Hz, 21-Me), 2.50 (1H, *brm* 3 β-H), 3.99 (2H, *d*, *J* = 4.5 Hz, $NHCH_2COOH$), 6.42 (1H, *t*, *J* = 4.5 Hz, $NHCH_2COOH$).

Glycoursodeoxycholic acid. Obtained from UDCA in 95% yield; m.p., 220–223°C (aq. EtOH) [lit. m.p., 228°C (4)]. IR ν_{max} cm^{-1}: 3343 (OH), 1633, 1723 (C=O), 1557 (CONH). ^1H NMR δ: 0.67 (3H, *s*, 18-Me), 0.93 (3H, *s*, 19-Me), 0.94 (3H, *d*, *J* = 6.3 Hz, 21-Me), 3.92 (2H, *d*, *J* = 5.4 Hz, $NHCH_2COOH$), 7.03 (1H, *t*, *J* = 4.5 Hz, $NHCH_2COOH$).

GCA. Obtained from CA in 92% yield; m.p., 143–145°C (aq. EtOH) [lit. m.p., 140–142°C (4)]. IR ν_{max} cm^{-1}: 3376 (OH), 1642, 1734 (C=O), 1560 (CONH). ^1H NMR δ: 0.68 (3H, *s*, 18-Me), 0.88 (3H, *s*, 19-Me), 1.00 (3H, *d*, *J* = 5.4 Hz, 21-Me), 3.96 (2H, *d*, *J* = 4.5Hz, $NHCH_2COOH$), 6.82 (1H, *t*, *J* = 4.5 Hz, $NHCH_2COOH$).

RESULTS AND DISCUSSION

Conventionally, condensation of a carboxylic group at C-24 in 5β-cholanoic acids with an amino group in taurine or glycinate ester has been attained by means of a mixed carbonic-carboxylic acid anhydride method in the presence of EEDQ as a coupling reagent and Et_3N as a catalyst (3,4,8,9). Our improved method reported here is the application of an alternative peptide coupling reagent, DEPC, for the amide bond formation (12). The amidation reaction involves the formation of intermediary acyl phosphates as carboxyl-activated compounds by interaction of carboxylic acids with DEPC in the presence of Et_3N in DMF and subsequent coupling with amines to give the desired amides.

Our initial effort was directed to determining optimal reaction conditions using DCA as a representative substrate. The yield of GDCA in the reaction product was determined as the methyl ester-trimethylsilyl ether derivative by capillary gas–liquid chromatography (11). The amidation reaction proceeded nearly quantitatively within 30 min by mixing DCA (1.0 eq.), glycinate ester (2.0 eq.), DEPC (1.2 eq.), and Et_3N (catalytic amount) in DMF at room temperature. The reaction is simple, clean, and rapid under the mild conditions and no undesirable by-products were formed . On the basis of the exploratory experiment, remaining unconjugated bile acids (LCA, CDCA, UDCA, and CA) were subjected to a similar amidation with glycinate ester (method B) and powdered taurine (method A) in the presence of DEPC to afford the corresponding glycine and taurine conjugates, respectively. All the reaction products showed a single spot on TLC.

Since the free acid forms of taurine-conjugated bile acids are extremely hygroscopic (4), they were converted to the sodium salts by treatment with NaOH after the amidation reaction (method A). To isolate the sodium salts of taurine conjugates as crystalline products, the reaction mixtures diluted with water were loaded on Sep-Pak C_{18} cartridges for reversed-phase solid extraction (13–15). The cartridges were washed successively with water and 20% aqueous EtOH to remove excess reagents, contaminants, and inorganic salts. Elution with EtOH gave the crystalline sodium salts of the desired taurine conjugates (taurolithocholic acid, taurochenodeoxycholic acid, tauroursodeoxycholic acid, taurodeoxycholic acid, and taurocholic acid) in excellent yield (89–93%) after recrystallization.

The use of a disposable C_{18} cartridge for solid extraction is therefore effective for rapid and easy purification of such polar and hydrophilic compounds, compared to a conventional adsorption chromatography on SiO_2 which is time-consuming, tedious, and needs large quantities of eluting solvent. A preliminary experiment for recovery and reproducibility tests by

a Sep-Pak C_{18} cartridge also revealed that nearly constant quantities of GCA are always retained on a Sep-Pak plus tC_{18} cartridge as described in the Experimental Procedures section: the approximate maximal retention capacity of bile acids is *ca.* 10%, relative to the sorbent mass of a Sep-Pak C_{18} used.

On the other hand, glycine-conjugated bile acids (glycolithocholic acid, glycochenodeoxycholic acid, glycoursodeoxycholic acid, glycodeoxycholic acid, and GCA) were isolated and purified as follows (method B): alkaline hydrolysis of the amidated bile acid methyl esters by treatment with methanolic KOH, followed by acidification with H_2SO_4, yielded the corresponding free acids. Direct recrystallization afforded analytically pure samples of the glycine conjugates of bile acids in high yield (92–96%). In the case of GCDCA, the alkaline hydrolyzed product of glycinated CDCA methyl ester was directly applied to a Sep-Pak C_{18} cartridge without acidification and obtained as the crystalline potassium salt, because the free acid was hard to crystallize (3).

In conclusion, this paper described a facile and efficient synthesis of biologically important taurine- and glycine-conjugated bile acids. The method may be utilized to prepare other uncommon amidated bile acids as reference compounds in relatively small-scale.

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A Synthesis of 4-Hydroxy-2-*trans*-nonenal and 4-(^{3}H) 4-Hydroxy-2-*trans*-nonenal

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ABSTRACT: 4-Hydroxy-2-*trans*-nonenal, the most abundant and toxic unsaturated aldehyde generated during membrane lipid peroxidation, was synthesized starting from fumaraldehyde dimethyl acetal. In the first step of the synthesis, the fumaraldehyde dimethyl acetal was partially hydrolyzed using amberlyst catalyst to obtain the monoacetal. The 4-hydroxy-2-*trans*-nonenal was synthesized by the Grignard reaction of the fumaraldehyde monoacetal with 1-bromopentane. 4-Hydroxy-2-*trans*-nonenal, obtained as its dimethylacetal, was oxidized to its corresponding 4-keto derivative using pyridinium chlorochromate buffered with sodium acetate as the oxidizing agent. 4-(^{3}H) 4-Hydroxy-2-*trans*-nonenal was obtained in one step by the sodium borotriteride reduction of the 4-keto derivative.
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Oxidative stress in biological systems, besides oxidizing proteins, results in the oxidation of the membrane lipids leading to an autocatalytic chain reaction of lipid peroxidation and formation of toxic unsaturated aldehydes (1). These metastable products of lipid peroxidation thus potentiate and spread oxidative damage initiated by the local and transient free radical events (1,2). Of the wide variety of aldehydes produced as a result of lipid peroxidation, 4-hydroxy-2-*trans*-nonenal (HNE) is by far the most abundant and cytotoxic (3). It belongs to a class of α-β unsaturated aldehydes which are generated by β-scission of alkoxyl radicals of n-6-polyunsaturated fatty acids such as linoleic or arachidonic acid (1,2). The extreme cytotoxicity of HNE is manifested in its diverse biological effects such as stimulation of neutrophil chemotaxis (1,4) and inhibition of several enzymes such as protein kinase-C, adenylate cyclase, and glyceraldehyde-3-phosphate dehydrogenase (5). Higher concentrations of HNE (40 to 60 μM) have genotoxic effects leading to sister chromatid exchange, micronuclei formation, and DNA fragmentation (6,7). At concentrations >100 μM, HNE and related aldehydes cause lethal toxicity by inhibiting glycolytic enzymes, mitochondrial respiration, and DNA and protein synthesis (1,8,9).

HNE can readily form Michael adducts with nucleophilic sulfhydryls or histidyl residue or form a Schiff base with primary amino groups of proteins (10–13). In recent years much effort has been directed to identifying the metabolic products of HNE and understanding its detoxification processes (1). The methods used for quantification of HNE in biological tissues are based mostly on high-performance liquid chromatography (HPLC) analysis of 2,3-dinitrofluorobenzene or 2,4-dinitrophenyl hydrazine derivatives of HNE (14). One method involves the use of 1,3-cyclohexanedione which gives a fluorescent dihydroacridine derivative, which can then be separated by HPLC (15). HNE has also been quantified by gas chromatography–mass spectrometry of the silyl derivative of its pentafluorobenzyl oxime (16). A major drawback of estimating HNE in biological samples by derivatization is the possible generation of HNE during the process of derivatization owing to autoxidation of polyunsaturated fatty acids (PUFA) or the breakdown of other oxidation products, thereby resulting in an overestimation of what was produced by the tissue (1). In this regard it would be useful to study HNE metabolism using a radiolabeled precursor since this would provide opportunity for quantification and identification of the metabolites.

The present methods available for synthesis of radiolabeled HNE are cumbersome. In one method, hexenal is reacted with 1,3-bis(methylthio)allyl-lithium in tetrahydrofuran at −75°C to give 3-bis(methylthio)-1-nonene-4-ol which is then converted to HNE by mercuric chloride. The overall yield is 47%. Radiolabeled HNE has been synthesized by using radiolabeled hexenal (17). HNE can also be synthesized by the reaction of the Grignard compound of propynal diacetal with hexanal to yield 4-hydroxy-2-nonynal diethyl acetal. The carbon-carbon triple bond is reduced to the double bond by the use of $LiAlH_4$ at low temperature (20°C). The diethyl acetal thus obtained is then hydrolyzed to HNE under mildly acidic conditions. When the tritiated analog of $LiAlH_4$ is used, tritiated HNE can be obtained. The yield of HNE is 53% by this method (Stephens, R.J., personal communication). A simple method of obtaining tritiated HNE is by sodium borotriteride reduction of the 4-keto derivative. However, a

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Abbreviations: HNE, 4-hydroxy-2-*trans*-nonenal; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

practical problem in developing this kind of synthesis is the difficulty in oxidizing the 4-OH group to yield the corresponding 4-keto derivative. One unpublished report obtained the 4-keto-derivative by oxidation with activated dimethylsulfoxide; however, this reaction has to be carried out at –70°C and takes almost 8 h for completion (18,19). We have developed a relatively simple method for substituting the 4H of HNE with tritium by the oxidation of the 4-OH group of the dimethyl acetal of HNE, to give the corresponding α-β unsaturated ketone, followed by reduction with sodium borotriteride. In this communication we report a synthesis of HNE and 4-(^{3}H) HNE starting from fumaraldehyde dimethyl acetal.

EXPERIMENTAL PROCEDURES

Amberlyst-15 ion-exchange resin, fumaraldehyde dimethyl acetal, pyridinium chlorochromate, chromic acid on anion exchange resin, 2,3-dichloro-4,5-dimethoxy benzoquinone, sodium acetate, 1-bromopentane, magnesium metal, dichloromethane (anhydrous), ether (anhydrous), and sodium carbonate were purchased from Aldrich Chemical Company (Milwaukee, WI). Sodium borohydride, sodium sulfate (anhydrous), ammonium chloride, acetone (HPLC grade) were from Fisher Scientific (Fairtown, NJ). Sodium borotriteride was purchased from DuPont NEN (Boston, MA).

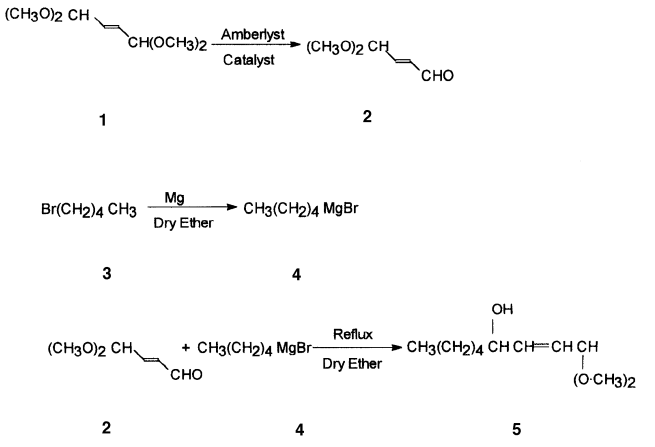
^{1}H spectra were recorded on a 270 MHz Jeol (Peabody, MA) Gx 270 and ^{13}C spectra on a 68 MHz Jeol apparatus, in ς units downfield from the internal standard Me_4Si.

All yields were calculated in percentage (moles) of the product obtained, relative to the number of moles of the starting parent compound.

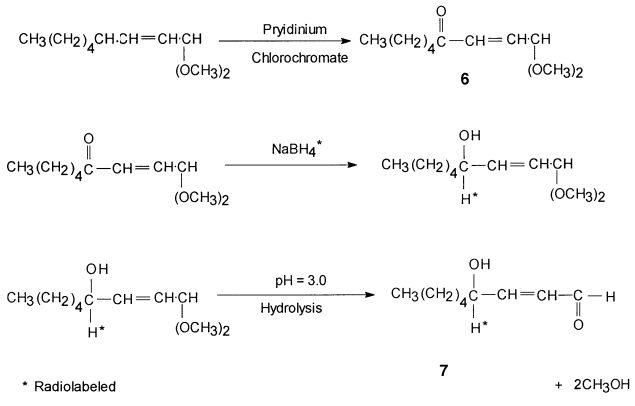
Nonradiolabeled HNE was synthesized from fumaraldehyde dimethyl acetal which was partially hydrolyzed to give the monoacetal using amberlyst-15 catalyst. The monoacetal was then reacted with 1-bromopentane, in a Grignard synthesis, which yielded HNE as its dimethyl acetal (20) (Scheme 1). HNE obtained as its dimethyl acetal was oxidized to its 4-keto derivative using pyridinium chlorochromate buffered with sodium acetate. The formation of the ketone proved especially tedious owing to the susceptibility of the HNE dimethyl acetal to acid hydrolysis even under mildly acidic conditions. The ketone, which is the oxidized product, once formed reverted easily to HNE and other degradation products (products not identified) upon exposure to moisture and higher temperature. A number of conventional oxidizing agents for the specific oxidation of allylic alcohols, as documented in the literature, were tried. However, most of the methods failed to yield the desired α-β unsaturated ketone. Pyridinium chlorochromate buffered with sodium acetate (1:1.2, w/w) was found to be the oxidizing agent of choice, and yielded the corresponding α-β unsaturated ketone (21) (Scheme 2). These results are summarized in Table 1. Other oxidizing agents led to the hydrolysis of the acetal to yield the aldehyde (HNE) as the major product, as determined by thin-layer chromatography (TLC). Although the synthesis of other 4-hydroxyalkenals is not reported, most of them can be synthesized by modification of the same general method described here, using the appropriate bromoalkane in the Grignard reaction.

Synthesis of fumaraldehyde monodimethyl acetal. The monoacetal **2** was obtained by the partial acid hydrolysis of the fumaraldehyde dimethyl acetal, using amberlyst-15 catalyst. In a typical reaction, 325 mL of acetone was stirred with 2.4 mL water and 3.26 g of amberlyst catalyst. To this mixture, 14.16 g (80 mmol) of the diacetal was added quickly in one portion, at room temperature. Stirring was continued for 6 min, after which the reaction mixture was filtered through a bed of anhydrous sodium carbonate. The solvent was distilled off under vacuum to yield fumaraldehyde monodimethyl acetal, a yellow oil (yield 8.4 g, 80%). The compound was found to be pure by observance of a single spot on TLC (2% methanol–dichloromethane), and by nuclear magnetic resonance (NMR). ^{1}H NMR ($CDCl_3$) ς (ppm) = 9.6 (*d*; 1H, –C*H*O), 6.7 (*dd*; 1H, CH = CH–CHO), 6.3 (*dd*; 1H, –C*H* = CH), 5.1 [*d*; 1H C*H*–$(OCH_3)_2$], 3.4 [*s*, 6H –$(OCH_3)_2$].

*Synthesis of dimethyl acetal of 4-hydroxy-2-*trans*-nonenal.* Magnesium pellets (2.8 g, 116.8 mmol) were added to 10 mL of dry ether. A few drops of 1-bromopentane were added, and the flask was gently heated until the reaction started. If no re-



SCHEME 1



SCHEME 2

TABLE 1
The Results of the Buffering of Pyridinium Chlorochromate with Sodium Acetate, Yielding the Corresponding α-β Unsaturated Ketone[a]

Oxidizing agent	Ratio of oxidizing agent to dimethyl acetal of HNE	Solvent and dilution	Reaction condition	Major product	4-Keto derivative of HNE
1. DDQ-dioxane	2 mol eq.	Dioxane 10 mg/mL	Stirred and refluxed for 6 h	HNE >90%	Not detected
2. Chromic acid on anionic exchange resin	3.5 g/mmole	Ether 5 mg/mL	Refluxed for 1 h	HNE >90%	<5% by HPLC
3. Activated manganese oxide	10:1 w/w	Hexane 10 mg/mL	Stirred at RT for 24 h	No reaction	
4. Pyridinium chlorochromate	1.5 millieq.	Dry methylene	Stirred at RT for 2 h	HNE >90%	Not detected
5. Pyridinium chlorochromate buffered with 1:1.12 w/w sodium acetate	1.5 millieq.	Dry methylene chloride 5 mg/mL	Stirred at RT for 30 min	Ketone 90% yield	90% yield

[a]DDQ, 2,3-dichloro-4,5-dimethoxy benzoquinone; HNE, 4-hydroxy-2-*trans*-nonenal; HPLC, high-performance liquid chromatography; RT, room temperature.

action was observed after heating, a crystal of iodine was added and the flask heated again, which was usually sufficient to initiate the formation of the Grignard reagent. Dry ether (150 mL) was then added to the reaction mixture followed by dropwise addition of the remaining amount of 1-bromopentane (13.2 g, 87.6 mmol) dissolved in 30 mL of dry ether. The reaction mixture was mildly heated so as to maintain gentle reflux for 30 min. At this stage, the mixture turned dark, as the formation of Grignard reagent proceeded to completion (Scheme 1). The reaction mixture was then cooled to 0°C by ice bath, and the monoacetal (9.5 g, 73 mmol), dissolved in 50 mL of dry ether, was added dropwise with constant stirring. When all of the monoacetal had been added, the stirring was continued for an additional 30 min at 0°C. Thereafter, the reaction was quenched with 75 mL of a saturated solution of ammonium chloride. It was extracted three times with ether, dried over anhydrous sodium sulfate, and the solvent was distilled off to give a brownish oil, the dimethyl acetal of 4-hydroxy-2-*trans*-nonenal. The crude compound was purified further by silica gel column chromatography in 3% methanol–dichloromethane. The purified compound moved as a single spot on TLC in 3% methanol–dichloromethane (yield 10.6 g, 72%). HNE can be obtaind by the acid hydrolysis (in 2.3% H_2SO_4) of the dimethyl acetal whenever required. The hydrolysis is complete in 1 h at room temperature. ^{1}H NMR ($CDCl_3$) ς (ppm) = 0.9 (3H, C*H*$_3$–CH$_2$), 1.2 (5H, C*H*$_2$–CH$_2$–C*H*–), 1.5 (2H, C*H*$_2$–CH$_2$–OH), 1.8 (1H, C*H*$_2$–C*H*–OH), 4.0 (*m*; 1H, C*H*–OH) 4.7 (*d*; 1H, C*H*–O), 5.6 (*dd*; 1H, CHOH–C*H*=CH), 5.9 (*dd*; 1H, C*H*=CH–CH(OCH$_3$)$_2$). ^{13}C NMR ($CDCl_3$) ς (ppm) = 14 (1C; *C*H$_3$–CH$_2$) 22–39 (4C; CH$_3$–*C*H$_2$–*C*H$_2$–*C*H$_2$–*C*H$_2$), 53 (2C; –(O*C*H$_3$)$_2$), 72 (1C, *C*H–OH), 103 (1C –*C*H–O) 126–138 (2C; *C*H=*C*H).

*Synthesis of 4-keto derivative of 4-hydroxy-2-*trans*-nonenal.* Pyridinium chlorochromate (800 mg, 3.71 mmol) was stirred with 1:1.12 w/w equivalent of sodium acetate, in 40 mL of dry methylene chloride. The dimethyl acetal of HNE (500 mg, 2.45 mmol) was dissolved in 10 mL of dry methylene chloride and added in one portion. The stirring was continued at room temperature for an additional 30 min, and the progress of the reaction was monitored by TLC, in 2% methanol–dichloromethane. At this stage the reaction mixture turned brownish due to the deposition of the reduced oxidizing agent. The reaction mixture was diluted with 250 mL (5 vol) of dry ether, the solvent was decanted, and the solid residue extracted three times with ether. The combined organic layer was filtered through a tight gauze of cotton, and the solvent was evaporated at reduced pressure. The ketone obtained was further purified by preparative TLC in 2% methanol–dichloromethane (yield 440 mg, 90%). The compound was found to be pure since it moved as a single spot on TLC (2% methanol–dichloromethane). ^{1}H NMR ($CDCl_3$) ς (ppm) = 1.0 (3H, C*H*$_3$–CH$_2$), 1.2–2 (6H, CH$_3$–C*H*$_2$–C*H*$_2$–C*H*$_2$), 2.5 (2H, C*H*$_2$–C = O), 3.5 (*s*; 6H, (COCH$_3$)$_2$), 6.0 (*dd*; 1H, CH=C*H*–CH) 6.3 (*dd*; 1H, O=C*H*–CH), 7.3 (*d*; 1H, CH–O) ^{13}C NMR ($CDCl_3$) ς (ppm) = 14 (1C, *C*H$_3$), 20–40 (4C, CH$_3$–*C*H$_2$–*C*H$_2$–*C*H$_2$–*C*H$_2$)$_m$ 102–110 (2C, *C*H=*C*H), 140 (1C, *C*H–O), 158, (1C, *C*=O).

*Preparation of 4-(^{3}H) 4-hydroxy-2-*trans*-nonenal.* The keto derivative (10 mg, 0.05 mmol) was suspended in 5 mL of absolute ethanol, 5 mCi (100–1000 mCi/mole) of sodium borotriteride was added, and the mixture was stirred for 2 h. Nonradiolabeled $NaBH_4$ (7.6 mg, 0.2 mmol) was then added and the stirring was continued for 2 h. The solvent was evaporated in a rotary evaporator and the reaction was quenched with 50 mL of acidified water (pH 2.0) to decompose the unreacted $NaBH_4$, extracted three times with methylene chloride, dried over anhydrous sodium sulfate, and the methylene chloride distilled off under vacuum. The crude compound thus obtained was purified by HPLC (yield 6 mg, 60%) as described below and eluted as a single peak (purity was 99%).

Purification of 4(^{3}H)-HNE by HPLC. The crude radiolabeled HNE was purified on a Rainin (Rainin Instrument, Woburn, MA) C_{18} ODS reverse-phase MicroSorb-MV col-

umn (4.6 mm × 25 cm) pre-equilibrated with 3% methanol (Solvent A), using a gradient with 50% methanol (Solvent B). The gradient was programmed such that B reached 100% in 30 min and was held at 100% for an additional 20 min, whereafter A reached 100% in 5 min. The absorbance was monitored at 224 nm. HNE eluted as a sharp peak at a retention time of 42 min. The radioactivity peak was found to coincide with this peak.

Determination of the specific activity. The concentration of HNE in the solution was calculated by determining the absorbance at 224 nm using a Gilford Response Spectrophotometer (Corning Laboratory Sciences, Oberlin, OH) and an extinction coefficient of 13,750 M^{-1} cm^{-1}. An aliquot of the solution was used for the quantification of radioactivity using a Beckman liquid scintillation counter model LS 1801 (Fullerton, CA). The purified 4(^{3}H) HNE had a specific activity of 10 mCi/mmole and a purity of approximately 99%.

RESULTS AND DISCUSSION

This method provides a simple synthesis for obtaining both HNE and 4-(^{3}H) HNE, starting from fumaraldehyde dimethyl acetal. The oxidation of HNE dimethyl acetal to its 4-keto derivative is conveniently achieved by oxidation with pyridinium chlorochromate buffered with sodium acetate. The ketone obtained thereby is reduced with sodium borotriteride to yield the 4-(^{3}H)-HNE. The oxidation reaction is performed at room temperature, is complete in 30 min, and results in high yields of the ketone (90%). Pyridinium chlorochromate alone failed to yield the desired compound resulting in the hydrolysis of the acid-labile acetal groups, and had to be buffered with sodium acetate 1:1.2 w/w. Although we tried other oxidizing agents, they did not yield the desired compound or gave a poor yield, and generally resulted in the hydrolysis of the acetal groups (Table 1).

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Chromogenic Assay for Phospholipase D from *Streptomyces chromofuscus*: Application to the Evaluation of Substrate Analogs

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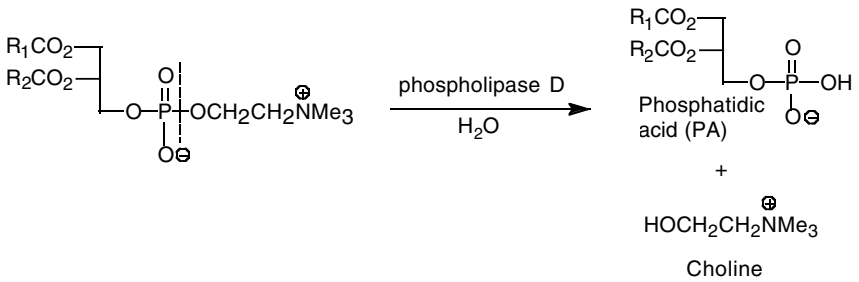
ABSTRACT: A rapid and convenient chromogenic assay for phospholipase D from *Streptomyces chromofuscus* (PLD_{Sc}) has been developed that converts the choline generated from the enzyme-catalyzed hydrolysis of phospholipids into a chromogenic dye. By quenching the reaction with EDTA at defined times, an initial rate curve is produced from which a k_{cat} and K_m can be readily derived. This assay has been applied to the biological evaluation of several substrate analogs, all of which appear to be activators rather than substrates or inhibitors of this enzyme. Performing the assay in 96-well microtiter plates allows for the easy screening of potential effectors of this enzyme. *Lipids 32,* 783–788 (1997).

Enzymes of the phospholipase D (PLD) class catalyze the hydrolysis of phosphatidylcholine (PC) to give phosphatidic acid (PA) and choline (Scheme 1). The intense interest in these enzymes is due to the discovery that the products of the PLD reaction *in vivo* serve as secondary messengers in the signal transduction cascade (1). Cellular PA can be converted to diacylglycerol (DAG) through the action of a PA phosphohydrolase (2), and the DAG that is produced then activates protein kinase C (3). The prolonged production of DAG through this PLD-mediated pathway represents an alternative to the rapid burst of DAG that is produced directly from the hydrolysis of phospholipids by phosphatidylinositol-specific phospholipases C (4). Moreover, there is evidence that PA directly activates a PA-dependent and DAG-independent protein kinase, which suggests a direct role for PLD in cellular signaling *via* protein kinases (5). Compounds that are specific activators and inhibitors of PLD would be extremely valuable as tools to probe the cellular function of this enzyme. 2,3-Diphosphoglycerate, a compound which possesses structural similarity to PLD product PA, is a competitive inhibitor of PLD (6). Although aminoglycoside antibiotics (7) and a chymotrypsin inhibitor (8) have been shown to inhibit PLD activity *in vivo*, the antibiotics lack specificity toward PLD alone, and the latter inhibitor does not appear to interact directly with PLD.

The cloning and purification of mammalian phospholipases D have proven difficult, whereas several PLD of bacterial origin have been cloned and isolated (9). These prokaryotic PLD have been accorded a greater significance owing to a recent report that compared the primary structures of PLD from numerous sources and found that bacterial and eukaryotic PLD have significant sequence similarity with one another (10). In addition, PLD isolated from various *Streptomyces* sources have been investigated in light of the observation that in the presence of an alcohol acceptor these PLD will catalyze a transphosphatidylation (11), a reaction that is synthetically useful (12). Of the bacterial PLD, the enzyme from *Streptomyces chromofuscus* (PLD_{Sc}) is one of the best characterized (13). This enzyme has been shown to be calcium-



SCHEME 1

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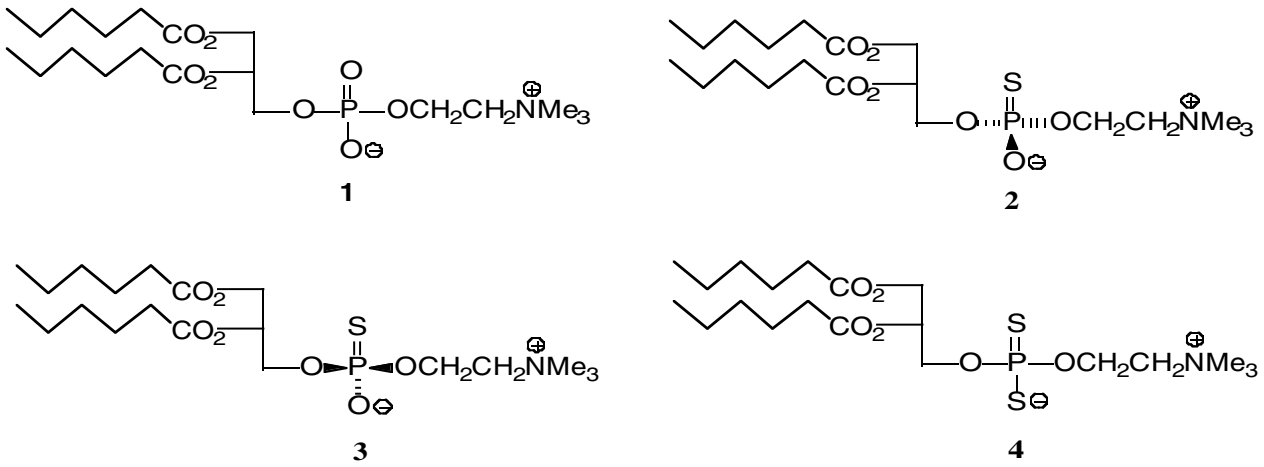
Abbreviations: CMC, critical micelle concentration; DAG, diacylglycerol; PA, phosphatidic acid; PAN, 1-(2-pyridyl(azo)-2-naphthol; PC, phosphatidylcholine; PLD, phospholipase D; PLD_{Sc}, phospholipase D from *Streptomyces chromofuscus.*



SCHEME 2

dependent and to possess a hydrophobic site that is distinct from its catalytic site (13). This bacterial PLD has been used as a model system for the mammalian enzyme (6), and compounds that activate or inhibit PLD_{Sc} may be useful for *in vivo* studies on mammalian systems. Analogs of the PC substrate **1** (Scheme 2) have been synthesized in which a single nonbridging phosphoryl oxygen has been replaced with sulfur, giving the R_p isomer **2** and the S_p isomer **3**; and in which both oxygens have been substituted by sulfur atoms, providing the dithioate compound **4** (14). We report an initial step toward deriving a structure–activity relationship for PLD_{Sc} using the substrate analogs **2**, **3**, and **4**.

To study the effects of various compounds on PLD_{Sc} activity, a sensitive assay was required that would utilize only small quantities of the substrate analogs. PLD assays using radiometric techniques (6) or safarine-based systems (15) have been reported, but these are too cumbersome for efficient screening of substrate analogs. Likewise, a chromogenic assay utilizing the substrate phosphatidyl-*p*-nitrophenol (16) was unattractive because of the concern that this compound did not adequately resemble the natural PLD substrate. Therefore, a known assay method for PLD_{Sc} that was based on the conversion of choline to a chromogenic dye through the action of choline oxidase, peroxidase, phenol, and 4-aminoantipyrine (17) was modified to a 96-well plate format to minimize the time and amounts of reagents required. We have recently described a variation of this assay (18) that was utilized to evaluate site-specific mutants of PC-preferring phospholipase C from *Bacillus cereus* (19).

MATERIALS AND METHODS

Enzymes. PLD from *S. chromofuscus* (P-8023), choline oxidase (C-5896), and peroxidase (P-8250) were obtained from Sigma Chemical Co. (St. Louis, MO).

Organic compounds. 1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). 1-(2-Pyridylazo)-2-naphthol (PAN) was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Substrate analogs. Substrate analogs were synthesized as described in Reference 14.

Assay of PLD—overview. Assays were carried out in 96-well microtiter plates that were maintained at 37°C using a constant-temperature water bath. A 50-µL solution of PLD_{Sc} in 100 mM Tris, pH = 8.0, with bovine serum albumin at 0.5 mg/mL was added to a 100-µL solution of the 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine substrate **1** in 50 mM Tris with 36 mM calcium chloride so that the final concentration of Tris was 67 mM, pH = 8.0. The final concentration of $CaCl_2$ was 24 mM and the final volume was 150 µL. The final concentration of PLD_{Sc} was 10.6 units/mL (3.5 units/150 µL reaction volume). The substrate concentrations typically ranged from 0.08 mM to 3.0 mM. After allowing the PLD_{Sc} reactions to progress for a defined time, the reactions were quenched with 75 µL of 0.1 M EDTA (pH = 8.0), followed by the addition of the chromogenic solution. The EDTA solution was added such that the final EDTA concentration was 25 mM. Quenching was performed at 20 s intervals to obtain points from $t = 0$ to $t = 100$ s. The chromogenic solution was added in a 75-µL volume and contained choline oxidase, phenol, 4-aminoantipyrine, and peroxidase in 60 mM Tris at pH = 8.0. This solution was added so that the final concentration of choline oxidase was 1.1 units/mL, phenol was 0.067 mg/mL, 4-aminoantipyrine was 0.1 mg/mL, and peroxidase was 3.5 units/mL. The increase in absorbance at 490 nm with time was monitored by a microplate reader (Bio-Tek Instruments EL311s; Winooski, VT) and found to come to an end point at $t = 60$ min.

Assay of PLD_{Sc}—detailed protocol. Table 1 shows the times at which the reagents in a typical assay were added. To prepare the microtiter plates for the assay, 50 µL of 0.1 M Tris at pH = 8.0 was added to the wells of a 96-well microtiter plate. A 50-µl aliquot of 3*x* substrate in water was then added, where *x* was the desired final concentration. Columns on the plate were used for the different substrate concentrations, and the rows represented the various times. The plate was then incubated in a 37°C bath for 10 min. After this equilibration to 37°C, 50 µL of the PLD_{Sc} solution (also pre-equilibrated to

TABLE 1
Time at Which Reagents Are Added in the PLD_{Sc} Assay[a]

Row on the 96-well microplate	Add PLD_{Sc} at time (s)	Add EDTA at time (s)	Time elapsed in PLD_{Sc} reaction (s)
5	10	110	100
4	20	100	80
3	30	90	60
2	40	80	40
1	50	70	20

[a]Assays were performed at 37°C in 96-well microtiter plates. Rows on the plates were used for the various assay times, with the plate columns representing different substrate concentrations. After the last addition of EDTA, the chromogenic solution (see the Materials and Methods section) was added to all wells of the plate. Row 6 was generally used as a blank, with EDTA being added before PLD_{Sc}. Abbreviation: PLD_{Sc}, phospholipase D from *Streptomyces chromofuscus.*

37°C) was added to the plate *via* a multichannel pipettor according to Table 1. Multichannel pipettors were used for all additions so that the hydrolysis reactions for each of the different substrate concentrations began and ended at exactly the same time. Mixing was achieved by pipetting 50 µL of the reaction mixture two times with the multichannel pipettor. As outlined in Table 1, the PLD_{Sc} reactions were quenched with 75 µL of 0.1 M EDTA at various times, which gave points in 20 s intervals from 20 to 100 s. Addition of EDTA was followed immediately by addition of 75 µL of the chromogenic solution. A blank in which the EDTA was added before the PLD_{Sc} solution was performed for each substrate concentration. The plate was then covered and incubated for 1 h at 37°C, at which time it was read at 490 nm. After subtracting the blank from each substrate concentration, conversion of absorbance at 490 nm to µmols choline was performed *via* the slope of the calibration curve shown in Figure 1. The slopes

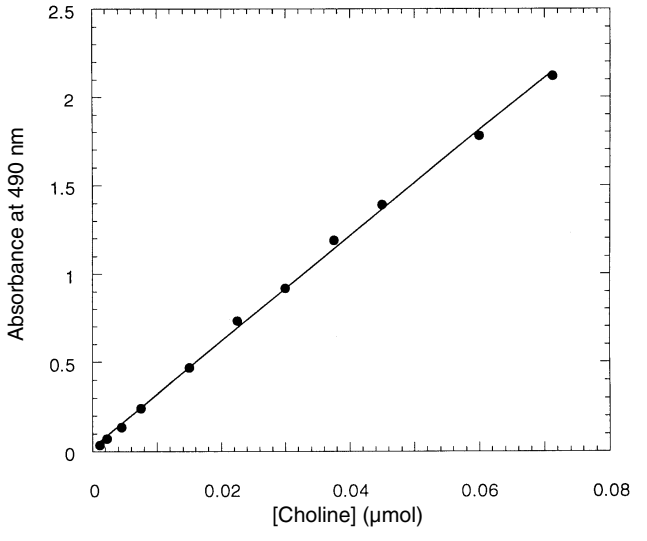


FIG. 1. Calibration curve for the phospholipase D from *Streptomyces chromofuscus* assay. Data points were generated by subjecting choline to the enzymatic reactions of the assay in microtiter plates at 37°C. The slope of this plot was used to convert absorbance at 490 nm to µmoles of choline produced.

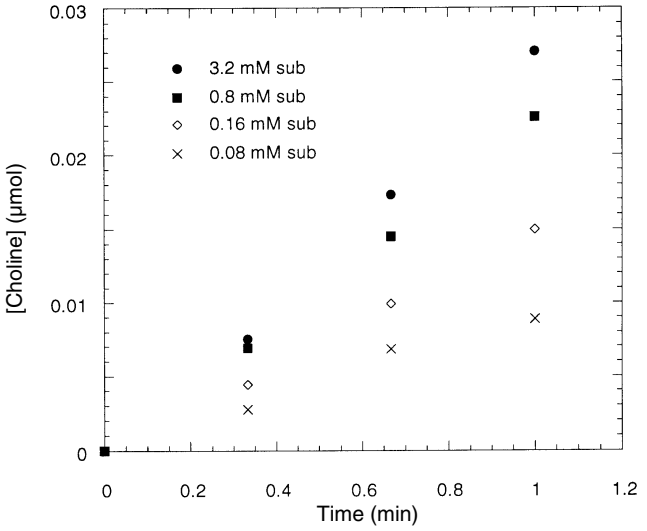


FIG. 2. Example of a plot used to obtain initial velocity values in the phospholipase D from *Streptomyces chromofuscus* assay. The slopes, in µmol/min, were taken from the linear portion of the graph.

of the resulting µmols of choline vs. time graphs (Fig. 2), which were generally linear through the 100-s point, were then plotted vs. substrate concentration as shown in Figures 3–5.

Values of K_m and V_{max} for the PLD_{Sc}-catalyzed hydrolysis of the PC substrate **1** were determined by a nonlinear least squares fit of the observed initial velocities (v) to Equation 1,

$$v = V_{max}\cdot[S]/(K_m + [S]) \quad [1]$$

using the KaleidaGraph curve-fitting program (Synergy Software, Reading, PA).

Assays of **2–4** *as potential substrates of* PLD_{Sc}. To determine whether compounds **2–4** were substrates for PLD_{Sc}, the

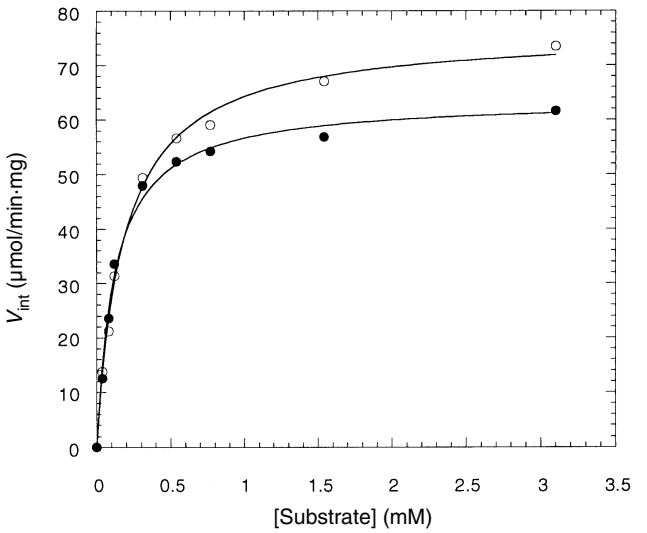


FIG. 3. Initial velocity (V_{int}) vs. substrate graph for the dihexanoylphosphatidylcholine **1** (●), and for **1** in the presence of compound **2** the R_p isomer of **1**, at 0.3 mM (○).

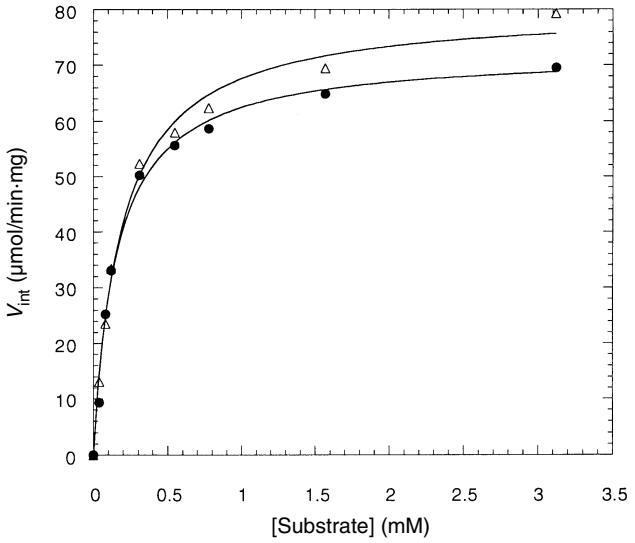


FIG. 4. V_{int} vs. substrate graph for the dihexanoylphosphatidylcholine **1** (●), and for **1** in the presence of compound **3** the S_p isomer of **1**, at 0.3 mM (△). See Figure 3 for abbreviation.

assay was performed as described above substituting these compounds for the substrate. Owing to the hydrophobic nature of the substrate analogs, the solutions containing the compounds were sonicated for 5 min prior to use to completely disperse the compounds. The concentrations at which the compounds were tested ranged from 0.03–0.7 mM for **2** and **3**, and 0.05 to 0.2 mM for **4**. Assays with **2**–**4** were allowed to proceed for as long as 4 min before quenching with EDTA, and no appreciable change in absorbance at 490 nm was measured over the blank.

Assays of **1** *in the presence of* **2**–**4** *and Triton X-100.* The effects of substrate analogs **2**–**4** and Triton X-100 on the

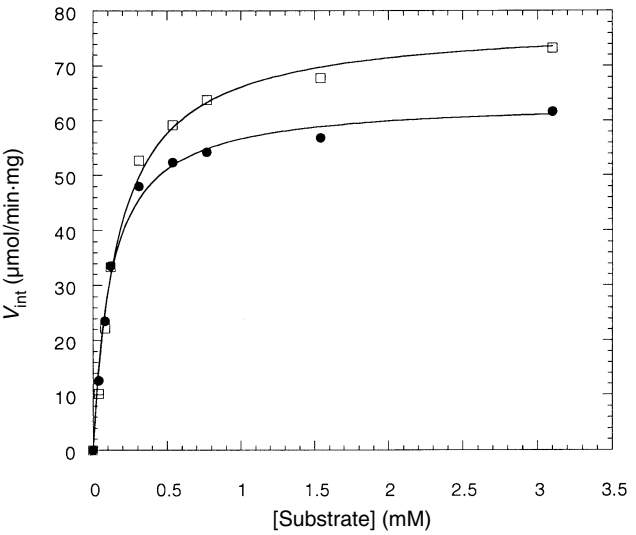


FIG. 5. V_{int} vs. substrate graph for the dihexanoylphosphatidylcholine **1** (●), and for **1** in the presence of compound **4** the dithioate compound of **1**, at 0.10 mM (□). See Figure 3 for abbreviation.

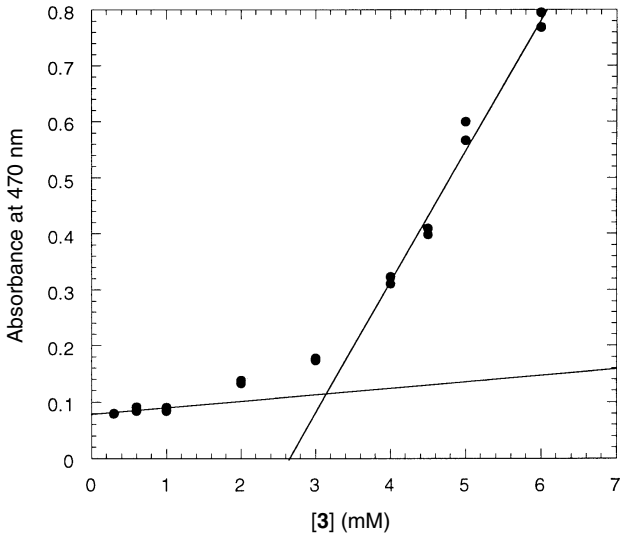


FIG. 6. Example of the graph used to determine the critical micelle concentration for compound **3**, the S_p isomer of dihexanoylphosphatidylcholine. Assays were performed in duplicate as described in the Materials and Methods section, and the critical micelle concentration was taken as the substrate concentration where the two lines intersected.

PLD_{Sc}-catalyzed hydrolysis of **1** were determined by performing the coupled chromogenic assay in the presence of varying concentrations of these compounds. The assays in the presence of **2**–**4** or Triton X-100 were performed by adding the substance in varying concentrations to the Tris buffer containing the PLD_{Sc} substrate **1**. Owing to the hydrophobic nature of the substrate analogs, these solutions were sonicated for 5 min prior to use. The assays were then performed as described above. The substrate analogues were tested in concentrations ranging from 0.1 to 0.3 mM for **2** and **3**, and 0.05 to 0.1 mM for **4**. The effect of Triton X-100 on the PLD_{Sc} reaction was studied at concentrations ranging from 0.03 to 0.2 mM of Triton X-100. The calibration curve shown in Figure 1 was obtained in the presence and absence of 0.2 mM **4** to control for any effects that these compounds may have on the enzymatic reactions converting choline to the chromogenic dye. The calibration curves were found to be identical in the presence and absence of **4** (data not shown).

Assays to determine critical micelle concentrations (CMC). Assays to determine the CMC for phospholipids **1**–**4** were run in duplicate on three different occasions. The assay was conducted in 96-well microtiter plates and is a modification of a known CMC assay based on the solubilization of the dye PAN (20). Varying concentrations of phospholipids **1**–**4** were made up to a total volume of 500 µL in H_2O. To each concentration was added 50 µL of a 1.6 mM PAN solution in pentane, at which point the pentane was allowed to evaporate and the color developed with an end point at 20 min. After 20 min, 200 µL of the solution was transferred to a 96-well microtiter plate and the absorbance was read at 470 nm. The absorbances obtained were graphed vs. substrate concentration and the CMC were calculated as described in Reference 20 and as shown in Figure 6.

RESULTS

Choline can be converted through the action of choline oxidase, peroxidase, phenol, and 4-aminoantipyrine into a chromogenic dye which has a strong absorbance at 490 nm (17). Addition of these reagents after quenching of the enzyme allows for formation of the dye, which is complete in 1 h. By quenching the calcium-dependent PLD_{Sc} reaction with EDTA at times from 20 to 100 s, a rate vs. time curve is obtained (Fig. 2). By following the assay plan shown in Table 1, an entire assay at eight different substrate concentrations can be performed in approximately 2 min. Conversion of absorbance at 490 nm to μmoles choline was performed *via* the calibration curve that was produced by subjecting choline to the enzymatic reactions of the assay (Fig. 1). The slopes (μmol/min·mg) of the rate vs. time plots (Fig. 2) were then graphed vs. substrate concentration as depicted in Figures 3–5. The kinetic parameters V_{max} and K_m were determined from a nonlinear least squares fit of the data to the Michaelis-Menton equation (Equation 1), and k_{cat} values were calculated using a molecular weight of 50,000 for PLD_{Sc} (13). The assay was typically performed with a PLD_{Sc} concentration of 10.6 units/mL; however, concentrations ranging from 2.65 to 21.6 units/mL were also used to confirm the linearity of the assay. The k_{cat} for PLD_{Sc} was $69 \pm 2\ s^{-1}$, with $K_m = 0.12 \pm 0.01$ mM. These kinetic parameters were alternatively obtained *via* the equation describing the Lineweaver-Burk transformation of the data. This analysis yielded a k_{cat} of $56 \pm 9\ s^{-1}$, and a K_m of 0.13 ± 0.04 mM.

Table 2 summarizes the kinetic data obtained for PLD_{Sc} with the PC substrate **1** and with the sulfur-containing analogs **2**–**4**. After it was found that compounds **2**–**4** were not substrates for PLD_{Sc}, they were tested for activation or inhibition of the enzyme. Figures 3–5 show the effect of the substrate analogs **2**–**4** on the PLD_{Sc} reaction. It is clear by comparing Figure 5 with Figures 3 and 4 that activation of PLD_{Sc} occurs at lower concentrations for **4** than for **2** and **3**. When **2** and **3** were tested at 0.1 mM, the activation of PLD_{Sc} was negligible (data not shown).

TABLE 2
Kinetic Parameters for Various Phosphatidylcholine Compounds in the PLD_{Sc} Assay[a]

Compound	k_{cat} (s^{-1})	K_m (mM)
1	69 ± 2	0.14 ± 0.02
2	No activity	No activity
3	No activity	No activity
4	No activity	No activity

[a]Assays were performed at 37°C in 96-well microtiter plates as described in the Materials and Methods section. The values of k_{cat} and K_m were determined from the nonlinear least squares fit of the initial velocities, *v*, to the equation, $v = V_{max} \cdot [S]/(K_m + [S])$, using the KaleidaGraph curve-fitting program. The k_{cat} values were calculated using a molecular weight of 50,000. Points for compounds **2**–**4** were taken out to 4 min, with no change in the absorbance at 490 nm over the blank detected. Abbreviations: **1**, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine substrate; **2**, R_p isomer of **1**; **3**, S_p isomer of **1**; **4**, dithioate compound of **1**.

CMC data (determined by measuring the amount of PAN solubilized as a function of phospholipid concentration) for compounds **1**–**4** with the 96-well microtiter plate PAN assay are as follows: **1**, 8.1 ± 0.6; **2**, 3.1± 0.2; **3**, 1.9 ± 0.5; and **4**, not available. This assay is displayed graphically in Figure 6, for phospholipid **3**. Of the four phospholipids, only compound **1** had its CMC determined previously. The CMC of **1** was found to be 11.1 mM by nuclear magnetic resonance methods (21), and this value differs from the 8.1 mM determined using the dye inclusion assay; however, these differences are relatively small and not surprising given the disparate nature of these two techniques. Compound **4** was insoluble at concentrations above 0.2 mM, and it did not show any appreciable change in the absorbance at 470 nm when analyzed in the PAN assay from 0.01–0.2 mM.

DISCUSSION

The rapid screening of stimulators/inhibitors of an enzyme requires an assay that will minimize the quantities of reagents required. This is especially important when using small organic compounds that require several synthetic steps to produce and enzymes that are costly or not commercially available. Assays performed in a 96-well plate format are ideally suited for such analysis because the small sample volume and the matrix format allow for rapid evaluation of the effect of selected organic molecules upon enzymatic activity. The present assay fulfills this objective and should find use not only for the screening of potential inhibitors of PLD_{Sc} but also for the determination of kinetic parameters for any site-directed mutants of the enzyme that may be produced in future studies of mechanisms.

Certain phospholipases have been shown to hydrolyze specifically one isomer of phosphorothioate analogs of phospholipids. For example, phospholipase A_2 from various sources is specific for the R_p isomer of these PC analogs (22–24). Conversely, the PC-preferring phospholipase C from *Bacillus cereus* specifically catalyzes the hydrolysis of the S_p thio isomer **3** of PC, whereas the R_p compound **2** is a potent inhibitor (14). Similarly, the PLD from cabbage was also found to be specific for the S_p isomer of thiophosphorylcholine (24). From the data presented in Table 2, it is obvious that neither the S_p nor R_p thiophosphates **3** and **2**, or dithio compound **4** are substrates for PLD_{Sc} under the conditions employed for the assay. This result suggests that both of the nonbridging phosphoryl oxygen atoms of the PC substrate are required for recognition and catalytic processing by PLD_{Sc}.

Close inspection of Figures 3–5 indicates that PLD_{Sc} is inhibited slightly by the substrate analogs **2**–**4** at low substrate concentrations but activated at higher substrate concentrations. This finding is similar to the effect upon PLD_{Sc} of Triton X-100, which caused inhibition at low substrate concentrations but activation at higher concentrations (13). The effect of Triton X-100 has been reproduced using this 96-well plate assay with the same results (data not shown). It has been suggested that PLD_{Sc} possesses a hydrophobic pocket that is

distinct from its active site, and activation of PLD_{Sc} by hydrophobic molecules has been described (13). The activation of PLD_{Sc} by hydrophobic compounds **2–4** is consistent with these results. Because many phospholipases are known to have increased activity in micellar systems (25), the assays studying the effect of **2–4** on the processing of **1** were carried out below the CMC of these compounds. Nevertheless, it is possible that the activation of PLD_{Sc} in the presence of **2–4** is due to pre-micellar aggregation of the lipids. Therefore, while it can be confidently concluded that **2–4** are not inhibitors of PLD_{Sc}, the mechanism of activation by these compounds remains uncertain.

A convenient assay for determining the kinetic parameters for PLD_{Sc} has been developed that is based on the conversion of choline to a chromogenic dye in 96-well plates. This assay has been applied to evaluate the phosphorothioate analogs **2–4** of PC, and it was found that these compounds are not substrates for the enzyme. Indeed these phospholipid analogs activate PLD_{Sc} at high substrate concentrations, with the more hydrophobic dithiophosphate analog **4** giving the most activation. In addition, a simple assay for the determination of CMC has been modified to fit a 96-well plate format, and this assay has been used to establish previously unknown CMC for **2–4**.

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COMMUNICATION

Structural Characterization of a "Signature" Phosphatidylethanolamine as the Major 10-Hydroxy Stearic Acid-Containing Lipid of *Cryptosporidium parvum* Oocysts

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ABSTRACT: A 10-hydroxy stearic acid-containing lipid from *Cryptosporidium parvum* was purified by thin-layer chromatography and analyzed by infrared spectroscopy, fast-atom bombardment mass spectrometry, ^{1}H and ^{31}P nuclear magnetic resonance spectroscopy, and was identified as phosphatidylethanolamine.
Lipids 32, 789–793 (1997).

Detection and removal of potentially pathogenic and nonculturable microorganisms are major problems in the water industry. Particularly alarming are the protozoal cysts of *Giardia* and oocysts of *Cryptosporidium* which are nonculturable with standard bacterial techniques, and are responsible for diarrhea worldwide (1,2). *Cryptosporidium parvum* is an important and serious pathogen because it infects a wide range of mammalian hosts and patients with acquired immune deficiency syndromes (3). The current methods available to detect oocysts of *C. parvum* in water are currently unsatisfactory (4).

The chemical analysis of oocysts based on lipids may provide a useful method for the sensitive and rapid detection of *C. parvum* (White, D.C., Alugupalli, S., Schrum, D.P., Kelly, S.T., Sikka, M.K., Fayer, R., and Kaneshiro, E.S., unpublished data). The importance of this type of chemical analysis to compare the lipid compositions of the *C. parvum* and Madin-Darby bovine kidney cells has previously been reported (3). In our recent studies, we have shown that the lipid analysis could differentiate between *C. parvum* and *C. muris* (White, D.C., Alugupalli, S., Schrum, D.P., Kelly, S.T., Sikka, M.K., Fayer, R., and Kaneshiro, E.S., unpublished data). The present study was undertaken in order to chemically characterize the ester-linked native form of 10-OH 18:0 in *C. parvum*.

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Abbreviations: FAB, fast-atom bombardment; GC/MS, gas chromatography/mass spectrometry; NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; TMS, trimethyl silylation.

MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade. *N,O*-Bis trimethylsilyl trifluoroacetamide, acetyl chloride, pyridine, and phospholipase A_2 (*Crotalus adamanteus* venom) were purchased from Sigma (St. Louis, MO). The standards 3-hydroxy tridecanoic acid and 6-hydroxy stearic acid were purchased from Matreya (Pleasant Gap, PA). The solvents hexane, chloroform, methanol, diethyl ether, and methylene chloride were also used.

Parasites. Oocysts of *C. parvum* used in this study were purified from calf feces as discussed previously (White, D.C., Alugupalli, S., Schrum, D.P., Kelly, S.T., Sikka, M.K., Fayer, R., and Kaneshiro, E.S., unpublished data).

Lipid extraction. Oocysts of *C. parvum* were extracted in a modified single-phase solvent system which included phosphate buffer (White, D.C., Alugupalli, S., Schrum, D.P., Kelly, S.T., Sikka, M.K., Fayer, R., and Kaneshiro, E.S., unpublished data; 5,6), at room temperature in chloroform/methanol/potassium phosphate buffer (50 mM, pH 7.4) (1:2:0.8, by vol) for 3 h. Chloroform and nanopure completely deionized, filtered water (1:1, vol/vol) were then added to cause a phase separation. The organic phase was then collected and dried under a stream of N_2 at 37°C.

Purification of lipids. The total lipid extract was dissolved in chloroform (~0.5 mL) and separated using a silicic acid column (10-cm column length, 0.5-cm inner diameter, 100–200 mesh silicic acid particle size) eluted with 5 mL of chloroform, 5 mL acetone, and 5 mL of methanol to elute neutral, glyco-, and phospholipids, respectively (7). Eluates were analyzed by silica-coated thin-layer chromatography (TLC) plates developed with chloroform/methanol/water (30:8:1, by vol). Sugar-containing compounds were visualized by spraying the plates with α-naphthol and heating at 110°C. Phospray reagent (Supelco, Bellefonte, PA) was used for revealing phosphorus-containing compounds, and ninhydrin reagent was used to reveal the presence of amino-containing species. Each individual lipid fraction was also ana-

lyzed for its fatty acid content. Final purification of the hydroxy fatty acid-containing lipid was achieved by reapplying the hydroxylated fatty acid-rich fractions on preparative TLC plates.

Selective phospholipase A_2 hydrolysis of phosphatidylethanolamine (PE) (8,9). Lyophilized phospholipase A_2 (250 units) was dissolved in 1 mL of 0.5 M Tris buffer with 0.4 M $CaCl_2$, at a pH of 7.5. A 300-µL aliquot of this solution was added to 0.4 mg of PE purified from *C. parvum* dissolved in 500 µL diethyl ether and shaken vigorously at 25°C for 20 min. The ether layer was removed, and the aqueous phase was washed with diethyl ether (2 × 1 mL). The ether washings were combined with the original ether layer, washed once with water (1 mL), collected, and dried under a stream of N_2 at 37°C. The fatty acids were subjected to acid methanolysis [1 M methanolic HCl (1 mL), 80°C, 30 min] and the hydroxy fatty acid methyl esters were separated, subjected to trimethyl silylation (TMS) and analyzed by gas chromatography/mass spectrometry (GC/MS) as previously described (10).

Other analytical techniques: (i) Characterization of the fatty acyl substituents. The acetone-eluted fraction from the silicic acid column was subjected to both mild and strong acid methanolysis as described previously (10). Fatty acid methyl esters were analyzed by GC/MS after the separation of hydroxy fatty acid methyl esters from nonhydroxylated compounds. The former class of fatty acid methyl esters was subjected to TMS derivatization prior to GC/MS analysis as described previously (White, D.C., Alugupalli, S., Schrum, D.P., Kelly, S.T., Sikka, M.K., Fayer, R., and Kaneshiro, E.S., unpublished data; 10).

(ii) Spectroscopy and spectrometry. Infrared spectra of samples were collected on a Bio-Rad FTS-60A Stepscan Interferometer (Bio-Rad Laboratories, Cambridge, MA) and measured as a thin film on NaCl plates.

^1H and ^{31}P nuclear magnetic resonance (NMR) spectra were obtained in $CDCl_3$ or $CDCl_3/CD_3OD$ (2:1, vol/vol) at 40°C on a modified NT360 NMR spectrometer (Nicolet, Madison, WI), operating at frequencies of 360.007 and 145.74 MHz, respectively.

Fast-atom bombardment (FAB) mass spectra were recorded with a ZAB-EQ (VG Analytical, Manchester, United Kingdom). Samples were dissolved in $CHCl_3$, and 1 µL was mixed on the probe tip with a matrix consisting of thioglycerol and 1% trifluoracetic acid. FAB was generated by an 8 keV xenon atom beam.

GC/MS was performed on a Trio-1 (VG Mass Lab) coupled to a Hewlett-Packard model 5890 gas chromatograph (Palo Alto, CA). Chromatographic separations were carried out on a fused-silica capillary column [30 m × 0.32 mm (inner diameter)] coated with cross-linked HP-1. Splitless injections were performed with a Hewlett-Packard model 7673 autosampler; the split valve was opened 1 min after injection. The carrier gas, helium, was used at an inlet pressure of 0.75 bar. The column temperature was programmed from 120 to 290°C, at 20°C/min; both the injector and the interface (between GC and MS) temperatures were kept at 280°C. The ionization was performed at 70 eV. The ion source temperature was 220°C in the electron impact mode. Hydroxy fatty acid methyl esters were identified and quantified as described previously (10).

RESULTS

Mass spectra. The electron impact mass spectrum of the TMS derivatized methyl ester ether (Me/TMS) of 10-OH 18:0 exhibited the characteristic fragments at *m/z* 215 (cleavage between C_9 and C_{10}), at *m/z* 273 (cleavage between C_{10} and C_{11}), and the molecular-specific ion at *m/z* 371 (M – loss of CH_3) (Fig. 1). Hydrolysis under both mild and strong conditions indicated that the 10-OH 18:0 is found mainly as ester-linked to the purified lipid.

Furthermore, the selective hydrolysis of PE purified from *C. parvum* with phospholipase A_2 indicated that 10-OH 18:O is located only on the *sn*-2 position as analyzed by GC/MS (data not shown).

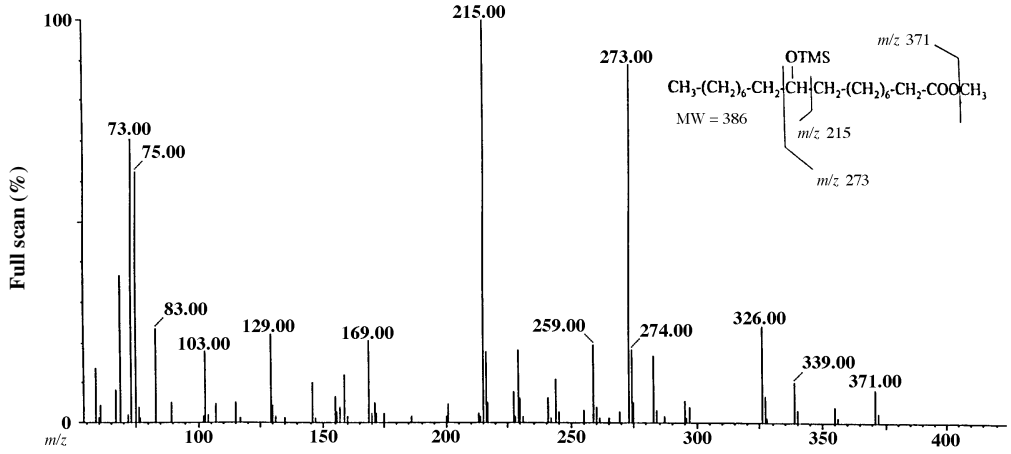

FIG. 1. Electron impact mass spectrum of the Me/trimethyl silylation (TMS)-derivatized 10-OH 18:0 found in *Cryptosporidium parvum*; MW, molecular weight.

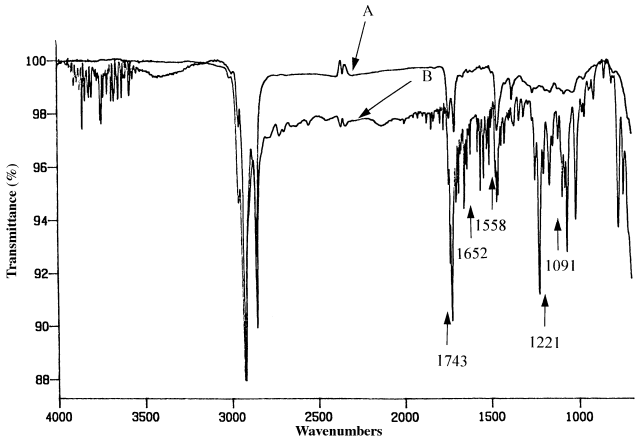

FIG. 2. Infrared spectrum of the purified phosphatidylethanolamine of *C. parvum* (marked A) and that of authentic phosphatidylethanolamine (PE) (marked B). The arrows indicate characteristic absorption bands typifying PE. See Figure 1 for abbreviation.

Fractionation of the total lipid extract by column chromatography followed by preparative TLC of the acetone-eluted fraction allowed the purification of the three lipids. The major lipid purified by preparative TLC was ninhydrin positive and phospray positive with the chromatographic mobility in one-dimension of authentic PE. This component lipid containing the ester-linked 10-OH 18:0 was also shown to be similar to authentic PE from its infrared, FAB/MS, ^{1}H NMR, and ^{31}P NMR spectra.

Figure 2 displays the infrared spectra of the major isolated lipid, which exhibited characteristic PE absorption bands at 1221 and 1091 cm^{-1} indicative for PO_2^-, 1652 and 1558 cm^{-1} indicative for NH_3^+ bands, 1743 cm^{-1} indicative of C=O bands, and 2850, 2920, and 2955 cm^{-1} characteristic of the aliphatic CH_3 and CH_2 stretching regions (between 2800 and 3000 cm^{-1}) (11). The positive FAB mass spectra of the native lipid (Fig. 3) showed the quasimolecular ion at *m/z* 664, confirming the PE structure and the presence of hydroxylated fatty acid substituents. The presence of ion at *m/z* 299 suggests that the lipid is acylated with hydroxylated stearic acid (12,13). Further confirmation of the lipid was also verified by ^{1}H and ^{31}P NMR to establish the configuration and the nature of the lipid (data not shown) (14).

DISCUSSION

The occurrence of 10-OH 18:0 in *Cryptosporidium* (White, D.C., Alugupalli, S., Schrum, D.P., Kelly, S.T., Sikka, M.K., Fayer, R., and Kaneshiro, E.S., unpublished data) led us to investigate the complex native form of this fatty acid in *C. parvum*. Comparision of the mild and strong methanolysis products has shown that 10-OH 18:0 occur in their native form mainly as an ester-linked lipid. Three lipids containing ester-linked 10-OH 18:0 were noted in the present study; however, the emphasis was placed mainly on the major lipid for structural characterization and molecular weight determinations. To the best of our knowledge, the present study is the first report of the presence of a 10-OH 18:0-containing lipid in *C. parvum*. The analysis reported herein shows that the extremely unusual fatty acid, 10-OH 18:0 which was previously reported in *Mycobacterium tuberculosis* lipids (15), is present in the PE of oocysts of *C. parvum* maintained under conditions where they retain infectivity for BALB/c neonatal mice.

ACKNOWLEDGMENTS

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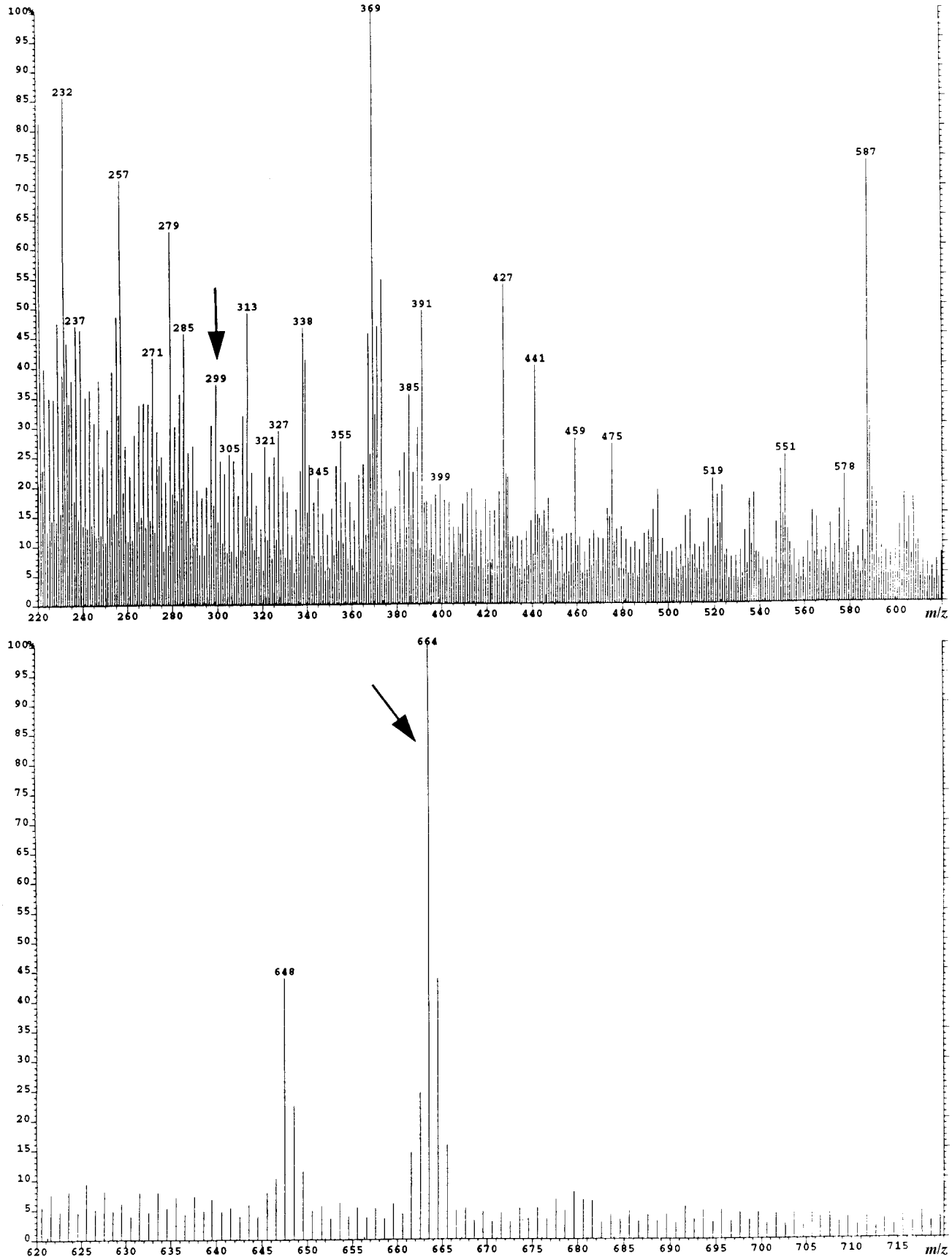

FIG. 3. Fast-atom bombardment-mass spectrum of the purified PE of *C. parvum*. Arrows point out molecular-ion peak ($[M + H]^+ = 664$) and peak corresponding to 10-OH 18:0. See Figures 1 and 2 for abbreviations.

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ARTICLE

Recombinant Antibody Fragments That Detect Enoyl Acyl Carrier Protein Reductase in *Brassica napus*

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ABSTRACT: Purified *Brassica napus* enoyl acyl carrier protein reductase (ENR) was used to select specific antibodies from a library of antibody fragments, single-chain F_v (scF_v), displayed on filamentous phage. Analysis of the selected clones by *Bst*NI fingerprinting and nucleotide sequencing showed that the scF_v were derived from three different human V_H germline genes. The binding specificities were confirmed by Western blots and ELISA. The scF_v preparations reacted with *B. napus* ENR, but not with β-keto reductase, nor enoyl reductase from *Escherichia coli*. Analysis of fragments generated by CNBr treatment indicates that the scF_v 3.13 recognizes an epitope located within the N-terminal 80 amino acids of the enzyme molecule. The scF_v were used to detect ENR directly in extracts of *B. napus* seeds.
Lipids 32, 805–809 (1997).

Lipids have many cellular roles: they provide structural components of membranes, anchor proteins to membranes, are involved in cell signaling and frost tolerance, and provide the correct biochemical environment for essential acylation and desaturation reactions (see Ref. 1 for review). From a commercial viewpoint, the ability of seeds to accumulate lipids as major storage compounds has been the main driving force in this area of research, together with the problems for the industry caused by large variation in fatty acids which occurs naturally in storage triglycerides. A detailed understanding of lipid biochemistry is an important prerequisite in any attempt to manipulate lipid composition. Although the basic pathways of lipid biosynthesis are now understood, the metabolic processes involved are very complex. Enoyl acyl carrier protein reductase (ENR) is part of the type II, dissociable, fatty acid synthetase system and catalyzes the NAD(P)H-dependent reduction of *trans*-Δ2-enoyl acyl carrier protein, an essential step in the *de novo* fatty acid biosynthesis. The activity of ENR increases continually throughout the lipid deposition phase in maturing seeds of oilseed rape *Brassica napus*. The native *B. napus* NADH-specific ENR has been purified and was shown to be a tetramer, consisting of subunits with a molecular mass of about 34 kDa. *Brassica napus* is an allo-tetraploid species containing two pairs of related ENR genes from its parents, *B. oleracea* and *B. campestris* (2). Sequence polymorphism between the parental ENR gene copies is minimal (2). Four isoenzymes of the enzyme have been identified by two-dimensional electrophoresis (3); they are present at different levels in the tissue. Isoforms II and IV are the most abundant in both leaf protein extract and seed material. *Brassica napus* ENR has been cloned and overexpressed in *Escherichia coli* (2). The crystal structure of an ENR-NAD complex has been solved at 1.9 Å resolution (4).

Studies on localization and quantification of ENR in plant tissues and purification of isoenzymes would be facilitated by the ready availability of specific antibody preparations. Polyclonal antisera have been raised against ENR, but the supply of affinity purified material is limited (5).

Large combinatorial phage display libraries have been produced, and a range of recombinant immune reagents with diverse specificities were isolated from such libraries (see Ref. 6 for review). The reagents are produced quickly without the need to immunize experimental animals, and the single-chain F_v genes can be genetically fused to reporter molecules or peptide tags for detection and affinity purification (7,8). Once an antibody fragment is selected from the library, the nucleotide sequences of the light- and heavy-chain variable regions are available for use in studies on plant biochemistry and metabolic processes by expressing the scF_v in plants. This paper describes the production and properties of scF_v specific for ENR by recombinant DNA techniques, and their use in detection of ENR in *B. napus* seeds.

EXPERIMENTAL PROCEDURES

Selection of ENR-specific scF_v from the phage antibody library. The Medical Research Council, United Kingdom (MRC) human synthetic scF_v library (9) was used for the selection of scF_v. In this library rearranged V_H genes were combined with a single Vλ3 chain in the phagemid pHEN. An amber mutation between the sequences encoding the scF_v and pIII means that strains of *E. coli,* that do not suppress the amber stop codon, secrete soluble scF_v (7). The scF_v have a

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Abbreviations: AP, Alkaline phosphatase; ECR, *Escherichia coli* enoyl reductase; ENR, enoyl acyl carrier protein reductase from *Brassica napus;* β-KR, β-keto reductase; PBS, phosphate-buffered saline; scF_v, single-chain F_v.

14-residue peptide (myc-tag) attached to the C-terminus which provides a means of detection and purification of soluble scF_v, using myc-tag specific monoclonal antibody 9E10 (10).

The library stock was amplified, and particles were rescued by superinfection with helper phage M13VCS (Stratagene), which supplies the viral genes to permit packaging of the phagemid DNA and the display of the scF_v-pIII fusion proteins on the surface of the phage particles.

For each round of selection, immunotubes (Nunc) were coated by incubation with a preparation of *B. napus* ENR at 60 μg/mL in 50 mM sodium carbonate buffer, pH 9.6, overnight at room temperature (approximately 22°C). The tubes were then rinsed with phosphate-buffered saline (PBS) and blocked with 2% nonfat dried milk (Marvel; Premier Beverages, Stafford, United Kingdom) in PBS. Phage preparations containing approximately 10^{12} colony-forming units were added to the tubes and incubated for 2 h at room temperature. After extensive washing, by rinsing the tubes 20 times with PBS containing 0.1% Tween 20 (BDH Laboratory Supplies, Merck Ltd., United Kingdom) and 20 times with PBS, bound phage was eluted with 100 mM trimethylamine, neutralized, and then used to infect *E. coli* TG-1 cells (suppressor strain). Phage particles were rescued by superinfection with helper phage M13VCS.

After the third round of selection, phage were rescued from single colonies of *E. coli* TG-1 by superinfection with helper phage. The resulting phage particles were collected by polyethylene glycol precipitation from the culture medium and tested by ELISA on antigen-coated plates.

Phage ELISA. Microtiter plates were coated with 10 μg/mL ENR, bovine serum albumin, cellulase, or macerozyme and incubated with phage preparations and then with polyclonal anti-M13 alkaline phosphatase (AP) conjugate (11). Bound AP was assayed by its reaction with *p*-nitrophenyl phosphate (Sigma, St. Louis, MO).

Soluble scF_v. Soluble scF_v were produced by inducing cultures of infected *E. coli* HB2151 (nonsuppressor strain) overnight at 25°C with 1 mM isopropyl-β-D-thiogalactopyranoside (Promega, Madison, WI) (7). Periplasmic extracts containing the scF_v were made from cell pellets by osmotic shock. Bacterial culture supernatant, containing secreted scF_v, was used directly in ELISA and Western blotting. Expression of the scF_v was verified using dot blots.

Sequencing of clones. Different clones, identified by their banding pattern in *Bst*NI fingerprinting (13), were sequenced by the dideoxy method using a USB sequencing kit and sequenase. The sequences were analyzed using GCG (14) and V BASE (15).

Western blot. Soluble scF_v were used in Western blots to detect ENR. One μg of the purified ENR was loaded per lane on SDS-polyacrylamide gels. *Brassica napus* extracts were made by grinding 30 seeds (approximately 35 d after flowering) in 2 mL of buffer (50 mM Tris-HCl, pH 6.8, 1 mM DTT, 0.1% Triton X-100) and centrifuging for 5 min. Twenty μL of the supernatant was loaded per lane. The seeds came from *B. napus* Jet Neuf and Dawson Park.

Related enzyme β-keto reductase (β-KR) (0.5 μg) from oilseed rape, the second reductase involved in fatty acid synthesis, and *E. coli* enoyl reductase (ECR) (0.34 μg) were used as control antigens. The samples were electrophoresed in a 12.5% polyacrylamide gel (16) and electroblotted onto nitrocellulose filters (17). The filters were blocked in 5% nonfat dried milk and the scF_v added. Incubation was for 4 h with gentle shaking at room temperature. After washing, the bound scF_v was detected with a mixture of the mouse monoclonal antibody 9E10 and an antimouse AP conjugate (Sigma). Enzyme substrate was 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma). Rabbit polyclonal antiserum raised against ENR (5) was used at a dilution of 1:4000, followed by antirabbit AP conjugate (Sigma).

Protein dot blots. ScF_v preparations from bacterial supernatant or periplasmic extracts were spotted directly onto nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) and air dried. The filters were blocked in 5% nonfat dried milk, and a mixture of mouse monoclonal antibody 9E10 and antimouse AP conjugate was added. Incubation was for 2 h with gentle shaking at room temperature. The enzyme substrate was 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

scF_v ELISA. Soluble scF_v preparations were tested in ELISA, either as periplasmic extracts or as bacterial culture supernatants. Microtiter plates were coated with ENR or control antigens (10 μg/mL in carbonate coating buffer) by incubation overnight at 4°C and then rinsed and blocked with 2% nonfat drid milk (Marvel) in PBS. The scF_v were mixed with the antibody 9E10 and added to the wells. After 3 h at room temperature, the plates were rinsed and incubated with antimouse AP conjugate (Sigma) followed by substrate.

Relative binding affinity. The method by Dyson *et al.* (18) was used. Dilutions of ENR were incubated with the phage-antibody preparation in solution until equilibrium was reached (18 h at 4°C). The amount of unbound phage remaining in solution was determined by adsorption on an ELISA plate coated with the substrate ENR. The adsorbed phage were then eluted from the solid phase and titered. The titer represents a value proportional to the free phage in the solution when the reactants are at equilibrium.

Epitope mapping. Purified ENR was digested with CNBr (19), the digest was separated by C_{18} reverse-phase high-performance liquid chromatography (20), and the fractions were spotted (2 nmole/spot) onto a polyvinylidene difluoride-based membrane (21). After wetting with methanol, the membrane was probed with scF_v as described for the Western blots.

RESULTS

Selection of scF_v. The numbers of input and output phage for each round of selection are shown in Table 1. The enrichment for specific binders is indicated by the increasing number of output phages after each consecutive round of selection.

After three rounds of selection and enrichment, 10 out of 14 individual clones tested exhibited binding specificities for

TABLE 1
Titers of Phage (cfu/mL) Obtained During Successive Rounds of Selection on Enoyl Acyl Carrier Protein Reductase

Selection round	Phage input	Phage output
1	5×10^{12}	1.56×10^{4}
2	4×10^{12}	1.2×10^{5}
3	5×10^{12}	1.9×10^{6}

ENR in phage ELISA (code names were 3.1, 3.2, 3.4, 3.7, 3.9, 3.10, 3.11, 3.12, 3.13, and 3.14). The DNA from these clones were subjected to *Bst*NI fingerprinting. This revealed two unique banding patterns (data not shown), suggesting that the V_H chain of the scF_v originated from different germline genes. This was confirmed by sequencing, which showed three groups of sequences, originating from different germline genes. The alignment of amino acid sequences from the scF_v in the three groups is shown in Figure 1. The heavy-chain families were compared with sequences in the V BASE sequence directory (15). ScF_v 3.13 belonged to human V_H 1 locus 1-02; the others belong to either V_H 3 locus DP 45 (3.2, 3.7, and 3.9) or locus DP 49 (3.1, 3.4, 3.10, 3.11, and 3.12).

ELISA. ELISA experiments using phage antibody were performed to identify binders obtained from the library by selection on antigen-coated tubes (Table 2). The absorbance values ($A_{405\ nm}$) range from 0.376 to 1.599. However, it is unlikely that the absorbance values obtained in the phage ELISA reflect the true binding affinity, since the number of phages harvested from small-scale *E. coli* cultures and the number of antibody fragments displayed on the surface of each phage particle vary. Soluble scF_v were produced by expression in the nonsuppressor strain HB2151 from the clones which gave high absorbance values in phage ELISA. Culture supernatants were tested for expression of scF_v using protein dot blots.

Four clones, 3.2, 3.7, 3.9 and 3.12, which reacted with ENR as phage antibodies, failed to react in ELISA when expressed as soluble scF_v. The positive results with these clones in phage ELISA may be due to the amplified detection of the phage by the anti-M13-AP conjugate, since the phage carries

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                                                    CDR 1
ER309   AQPAMAEVQLVESGGGLVQPGGSLRLSCAGSGFTFSSYAMHW
ER310   AQPAMAEVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHW
ER313   AQPAMAQVQLVQSGAEVKKPGASVKVSCKASGYTFTGYYMHW

                          CDR 2
ER309   VRQAPGKGLEWVSAIG-TGGGTYYADSVKGRFTISRDNAKNS
ER310   VHQAPGKGLEWVAVISYDGSNKYYADSVKDRFTISRDNSKTT
ER313   VRQAPGQGLEWMGWINPNSGGTNYAQKFQGWVTMTRDTSITS

                                    CDR 3
ER309   LYLQMNSLRAEDTAVYYCARGTRSVTSVTLAWGQGTLVTVSR
ER310   LYLQMNSLRAEDTAVYYCAR------KYVFGWGQGTLVTVSR
ER313   AYMELSRLRSDDTAVYYCAR----VMTPF--WGQGTLVTVSR
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FIG. 1. Alignment of amino acid sequences of anti-enoyl acyl carrier protein reductase from *Brassica napus* (ENR) scF_v heavy chains derived from three different germline genes. The complementary determining regions (CDR) are shown in boxes. ER: enoyl reductase.

TABLE 2
Absorbance Values ($A_{405\ nm}$) Obtained in PTA-ELISA of Phage Particles Against Purified Plant ENR and Cellulase[a]

Phage clone	Purified plant ENR	Cellulase	Carbonate buffer
3.1	1.263	0.076	0.141
3.2	1.507	0.075	0.092
3.4	1.599	0.066	0.108
3.7	0.521	0.082	0.092
3.10	0.376	0.069	0.061
M13VCS[b]	0.057	0.067	0.072

[a]Absorbance values measured after 1 h incubation with substrate. ENR, enoyl acyl carrier protein reductase from *Brassica napus;* PTA, plate-trapped antigen.
[b]Helper phage only.

about 2700 copies of its major coat protein pVIII against which the polyclonal antiserum would be mainly directed (9). The binding of the soluble scF_v to ENR was shown to be highly specific. No cross-reaction was detected when ECR or β-KR was used at 10 μg/mL to coat ELISA plates (Table 3).

Moreover, we found that the soluble scF_v preparations of the recombinant antibodies 3.10 and 3.13 remained active for at least 6 mon, and the bacterial culture supernatants were functional in ELISA at dilutions of up to 1:100 (data not shown).

Western blotting. Soluble scF_v 3.1, 3.4, 3.10, and 3.13 reacted in immunoblots with SDS-treated purified preparations of ENR. The binding properties of scF_v 3.10 and 3.13 were further investigated; between 0.3 and 1 μg of each ENR, β-KR or ECR, and also crude extracts of *B. napus* seeds in Laemmli sample buffer (20 μL per lane) were tested. Neither scF_v 3.10 nor scF_v 3.13 reacted with β-KR or ECR in the immunoblots (Fig. 2A and data not shown for scF_v 3.10), but detected purified plant ENR and ENR in *B. napus* seed extracts. The additional bands detected in the purified ENR sample are most likely degradation products. When polyclonal antiserum raised against ENR was used to probe an immunoblot, the additional bands in the purified ENR sample were also present (Fig. 2B). The presence of β-KR and ECR was verified by Coomassie-staining of the gel (for ECR) or probing with polyclonal antiserum raised to β-KR. Bands detected had the appropriate molecular mass of about 28 kDa (Fig. 3).

Affinity. The binding affinity of scF_v 3.13 anti-ENR antibody was measured using the method of Dyson *et al.* (18). This method allows the determination of relative dissocia-

TABLE 3
Absorbance Values ($A_{405\ nm}$) Obtained in PTA-ELISA of Soluble scF_v Fragments Against ENR, ECR, and β-KR[a]

scF_v	Plant ENR	ECR	β-KR	Carbonate buffer
3.10	0.508	0.057	0.055	0.059
3.13	0.643	0.055	0.056	0.053
TY[b]	0.055	0.057	0.056	0.055

[a]Absorbance values measured after 1 h incubation with substrate. ECR, *Escherichia coli* enoyl reductase; β-KR, β-keto reductase; scF_v, single-chain F_v. See Table 2 for other abbreviations.
[b]*Escherichia coli* growth medium only.

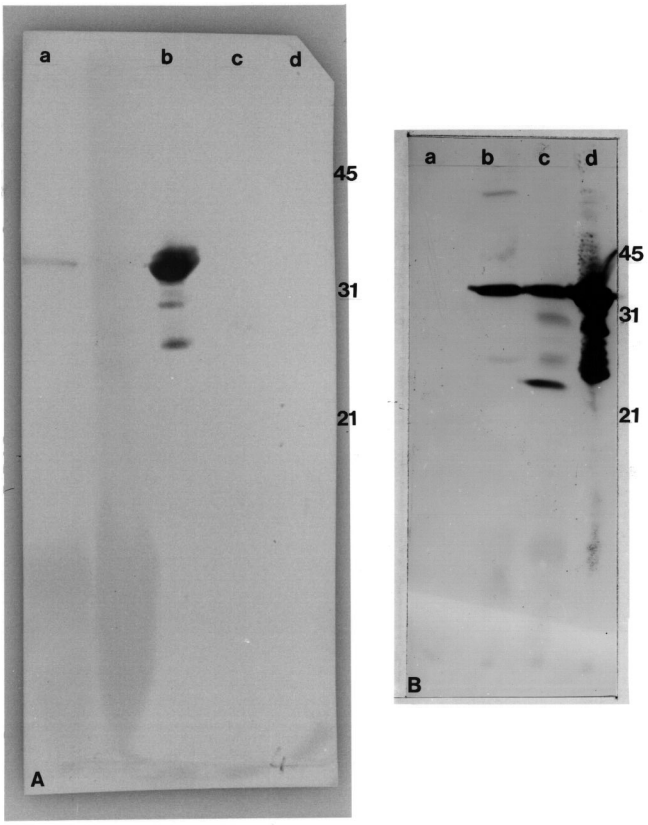

FIG. 2. A: Western blot probed with single-chain F_v 3.13. Tracks contain: a: *Brassica napus* seed extract (20 µL); b: purified ENR (1 µg); c: purified β-keto reductase (β-KR) (0.34 µg); d: purified *Escherichia coli* reductase (0.5 µg). B: Western blot probed with polyclonal antiserum raised against ENR (1:4000). Tracks contain: a: purified β-KR (0.34 µg); b: *B. napus* seed extract (20 µL); c: *B. napus* leaf extract (20 µL); d: purified ENR (1 µg). The numbers to the right indicate the position of the molecular mass markers (kDa). See Figure 1 for other abbreviations.

tion constants (K_d^{Rel}) between filamentous phage carrying fusions to the coat protein pIII and substrate (ENR) in solution. We determined K_d^{Rel} for scF_v 3.13 as 1.6×10^{-8} M. This value represents a moderate affinity, and is commonly found for scF_v from primary phage repertoires (22).

Epitope mapping. Purified ENR was digested with CNBr, the digest was separated by C_{18} reverse-phase high-performance liquid chromatography, and the fractions were spotted onto polyvinylidene difluoride-based membrane and probed with scF_v 3.13. Only one of the fractions in the dot blot reacted with scF_v 3.13 (data not shown). The amino acid sequence of this fraction was determined, and the epitope was deduced to be within the 80 amino acids of the N-terminus of the ENR molecule.

DISCUSSION

The results show that a set of scF_v specific for ENR were obtained from a phage display library of antibody variable regions after only three rounds of selection. The length of the CDR 3 of the heavy chain of the different anti-ENR scF_v ranged from 5 to 11 amino acids, thus covering the range possible with the MRC human synthetic scF_v library, where the heavy chain CDR 3 was designed to consist of 4 to 12 amino acids (9).

The binding properties of two of the scF_v, 3.10 and 3.13, were studied in more detail. The scF_v were highly specific. They reacted with ENR in ELISA and immunoblotting tests, but not with β-KR which belongs to the same biosynthetic pathway and which shares 25% sequence identity with ENR (23), or ECR.

The scF_v 3.13 epitope was located within the N-terminal 80 amino acids of the ENR molecule. The antigenic index calculated according to Jameson and Wolf (14) indicates that the N-terminus of ENR is antigenic (index >1.0 for nine amino acids at the N-terminus), whereas a similar plot for the N-terminus of β-KR gave values of only –0.45 to 0.45 for the first six amino acids. These data together with the experimental finding suggest that the epitope recognized by scF_v 3.13 is at the mobile N-terminus of the ENR molecule.

Within the N-terminal 80 amino acids of ENR, only two amino acid differences have been detected by Kater *et al.* (2) between the amino acid sequence deduced from nucleotide sequencing data and sequence data determined by direct peptide sequencing of purified ENR protein, reflecting minor sequence polymorphism between the parental ENR genes. The results from Southern blots also suggest a very high degree of sequence homology between the two ENR genes found in members of the Cruciferae family (2). It is therefore highly likely that scF_v 3.13 will react with all isoforms of the enzyme, and it may be useful in affinity purification of the

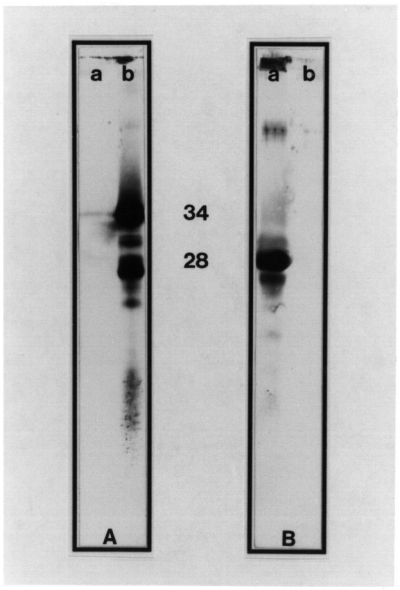

FIG. 3. A: Western blot probed with polyclonal antiserum raised to ENR (1:4000). B: Western blot probed with polyclonal antiserum raised to β-KR (1:4000). Tracks contain: a: purified β-KR (1.7 µg); b: purified ENR (1 µg). The numbers indicate the molecular mass in kDa. See Figures 1 and 2 for abbreviations.

isoenzymes.

In this paper we have demonstrated the selection from a phage display library of scF_v binding to the plant enzyme ENR. The scF_v will be used in studies to facilitate the understanding of plant lipid biosynthesis and its regulation. The expression of the anti-ENR scF_v genes in transgenic plants may provide a useful alternative to antisense approaches to study and influence biosynthetic pathways during lipid biosynthesis. ScF_v present an attractive tool for intracellular immunomodulation of plants. Plants transformed with antibody genes have been shown to express functional proteins in the cytosol (24,25), and the antibodies can be successfully targeted to specific plant organs, e.g., roots (26) or seeds (12).

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Use of Cyclodextrin to Deliver Lipids and to Modulate Apolipoprotein B-100 Production in HepG2 Cells

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ABSTRACT: 2-Hydroxypropyl-β-cyclodextrin (cyclodextrin), cyclodextrin-solubilized oleate, and cyclodextrin-solubilized cholesterol were used to modulate proteolysis and secretion of newly-synthesized apolipoprotein B-100 (apoB) in HepG2 cells. Following cyclodextrin and lipid treatments, cells were pulse-labeled with [^{3}H] leucine, and quantitative immunoprecipitation was used to measure apoB synthesis, apoB secreted into the medium, and the cellular content of undegraded apoB that was not secreted. Three-hour treatment with cyclodextrin-solubilized oleate (0.2 mM) increased secreted apoB from 4% (control cells) to 32% and cellular undegraded apoB from 15% (control cells) to 64% of apoB synthesized, which is consistent with earlier studies using bovine serum albumin to complex exogenous oleate. Prolonged daily (4 d or more) administration of 0.5% (3.5 mM) cyclodextrin with medium containing 10% fetal bovine serum increased the secretion of nascent apoB from 5–10% (control) to 17–28% and cellular undegraded apoB from 15–20% (control) to 25–31% of apoB synthesized, respectively. Subsequent administration of cyclodextrin-solubilized cholesterol (10–40 μg) for only 3 h reversed the cyclodextrin-mediated increase in apoB secretion. The application of 0.5% cyclodextrin to HepG2 cells can rapidly (within minutes) stimulate cholesterol efflux, and transiently (over a 1–2 d period) increase cholesterol synthesis. In the current studies, the cyclodextrin-mediated increase in cholesterol synthesis was not concurrent with the increase in apoB secretion. However, prolonged (15 d) administration of cyclodextrin was shown to increase the cellular free cholesterol concentration by 25–41%, reduce the cellular triglyceride concentration by 59%, and increase apoB secretion 3- to 4-fold, without affecting the cellular cholesteryl ester concentration. In comparison, 14-d treatment with cyclodextrin-solubilized cholesterol (20 μg/mL) followed by 1-d equilibration without cholesterol was shown to increase the cellular free cholesterol and cholesteryl ester concentrations by 76% and 10-fold, respectively, although apoB secretion was not affected. It is hypothesized that chronic daily administration of 0.5% cyclodextrin increased the cellular cholesterol concentration and flux in discrete putative regulatory compartments, which "shielded" nascent apoB from rapid proteolysis and facilitated apoB secretion. In conclusion, cyclodextrin was used independently and in combination with cholesterol or oleate to modulate apoB proteolysis and secretion. We speculate that subcellular changes in cholesterol concentration and flux may modulate apoB production in HepG2 cells.

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The primary function of very low density lipoprotein (VLDL) is transport of newly-synthesized triglyceride from liver to peripheral tissues. In the capillary beds, plasma VLDL are converted to cholesteryl ester-enriched triglyceride-poor low density lipoprotein (LDL) (1,2). Elevated plasma LDL and apolipoprotein B-100 (apoB) concentrations are primary risk factors for the development of atherosclerosis and coronary heart disease (3–5). Many of the chronic hyperlipoproteinemias associated with atherosclerosis result from hepatic overproduction of apoB-lipoproteins, predominantly VLDL (6–12). However, the mechanisms regulating hepatic apoB-lipoprotein secretion, and specific roles played by cholesterol (CH) metabolism, are not completely understood.

ApoB-100 is synthesized in hepatocytes as a 550 kD glycosylated protein (13–15) that cotranslationally associates with the membrane of the endoplasmic reticulum (ER) (16–19). Complete translocation of nascent apoB across the ER membrane into the ER lumen and apoB transport in the secretory pathway require the complexation of lipid substrates with apoB at the lumenal side of the ER (17,20). An increase in triglyceride synthesis has generally been considered to be a potent stimulus of apoB-lipoprotein assembly and secretion (21–23). However, roles played by the ER CH content, CH biosynthesis, the cellular cholesteryl ester concentration, and CH flux across the liver have not been clearly demonstrated. Some studies have suggested that the hepatocellular cholesteryl ester concentration is an important regulator of apoB-lipoprotein secretion (24–28); whereas, others have indicated no effect of cholesteryl ester on apoB production (29,30). The rate of association of lipid substrates with nascent apoB and the rate of translocation of apoB-lipoprotein particles across the ER membrane into the ER lumen determine whether nascent apoB will be transported in the secretory pathway or proteolytically degraded shortly after translation (31–36). Secretion of apoB-lipoprotein is depen-

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; apoB, apolipoprotein B-100; BSA, bovine serum albumin; cyclodextrin or CD, 2-hydroxypropyl-β-cyclodextrin; CH, cholesterol; ER, endoplasmic reticulum; FBS, fetal bovine serum; HDL, high density lipoprotein; HMG-CoA, β-hydroxy-β-methylglutaryl CoA; LDL, low density lipoprotein; MTP, microsomal triglyceride transfer protein; OA, oleate; PBS, phosphate-buffered saline; VLDL, very low density lipoprotein.

dent upon the expression of microsomal triglyceride transfer protein (MTP) (37–39), which has been suggested to transport both triglyceride and cholesteryl ester from the cytosolic surface of the ER into the ER lumen (40). Recently, MTP has been shown to associate with nascent apoB during early stages of apoB-lipoprotein assembly in cultured HepG2 cells (41).

The human hepatoblastoma cell line HepG2 is commonly utilized as a model system for studying hepatic lipoprotein production (42,43). These cells retain a variety of liver-specific functions, including synthesis of apoB and secretion of apoB-lipoproteins (44–48). Investigation of the association between CH metabolism and apoB-lipoprotein secretion requires independent modulation of cellular CH metabolism. Exogenous CH is commonly administered to cultured hepatocytes through the addition of liposomes, lipoproteins, or a CH-ethanolic solution to the culture medium. However, liposomes and lipoproteins can alter the cellular content of lipids other than CH, and lipoproteins enter the cell mainly through highly-regulated receptor-mediated processes that limit the efficiency of cellular CH uptake. Since ethanol has been shown to independently affect apoB metabolism (49,50), it is an undesirable choice to solubilize CH for apoB studies.

In the current study, 2-hydroxypropyl-β-cyclodextrin [cyclodextrin (CD)] was used as a research tool to study apoB production in HepG2 cells. Cyclodextrin is a water-soluble oligosaccharide consisting of a cyclic hydroxypropyl-substituted backbone of seven α-1,4-D-glucopyranose units (51). The hydrophobic interior cavity forms an inclusion complex with CH (52) that greatly increases CH solubility (53). The current study measured the effect of prolonged chronic treatment with 0.5% (3.5 mM) CD on rapid apoB proteolysis in classical pulse-chase experiments that used [^{3}H] leucine to label newly synthesized apoB. CD was also used to deliver CH and to increase the cellular CH concentration. Finally, we demonstrated the use of CD-solubilized oleate (OA), instead of bovine serum albumin (BSA)-solubilized OA, to reduce apoB proteolysis and to increase apoB secretion. The results may provide a foundation for additional cell culture studies that examine the effects of changes in hepatic subcellular lipid concentrations and flux on apoB-lipoprotein production.

MATERIALS AND METHODS

Materials. L [4,5-^{3}H] Leucine (5 mCi/mL, 131 Ci/mmol) and [2-^{14}C] Na-acetate (200 μCi/mL, 56 mCi/mmol) were purchased from Amersham Corporation (Arlington Heights, IL). 2-Hydroxypropyl-β-cyclodextrin (avg. molar substitution = 0.6, avg. MW = 1380) was purchased from Aldrich Chemical Co. (Milwaukee, WI). CH, Na OA, tissue culture-grade collagen, fetal bovine serum (FBS), and protein assay kit #P5656 were obtained from Sigma Chemical Co. (St. Louis, MO). Eagle's minimal essential medium, nonessential amino acids, Na pyruvate, penicillin, and streptomycin were purchased from Gibco BRL Life Technologies (Grand Island, NY). Leucine-free medium was generated from a minimum essential medium select-amine kit (Gibco catalog #300-9050AV). Leupeptin and pepstatin were obtained from Peninsula Laboratories, Inc. (Belmont, CA). Protein A-sepharose CL-4B beads were from Pharmacia LKB Biotechnology, Inc. (Alameda, CA). Polyclonal anti-human apoB antibody was raised in a rabbit. Sheep antihuman albumin was purchased from Harlan Bioproducts for Science, Inc. (Indianapolis, IN). Other chemicals and reagents were of the highest purity available.

Culturing of HepG2 cells. HepG2 cells were seeded into Falcon tissue culture plates (9.6 cm^2/well) or dishes (60 mm diameter) pre-coated with collagen. Cells were grown in humidified air containing 5% CO_2 at 37°C. Cells were incubated in either complete growth medium (Eagle's minimum essential medium with: 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM Na pyruvate, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin), serum-free medium (SFM) (complete medium without 10% FBS), or serum-free leucine-free medium (SFLFM) (used for the pulse-labeling periods only). Unless noted, fresh medium was administered every 48 h. Cells were subjected to various treatment protocols as described in the figure legends and tables.

Preparation and application of CD, CD-solubilized OA, and CD-solubilized CH solutions. CD was dissolved with stirring in phosphate-buffered saline (PBS), generally at a concentration of 2 g/10 mL (20%, wt/vol). The appropriate amount of CH or Na OA was dissolved with stirring into the CD solutions under a gentle stream of N_2. Dissolution of OA was rapid at room temperature; however, solubilization of CH required warming to 40–50°C for 10–15 min. Solutions were passed through a 0.2-μm filter (Sterile Acrodisc; Gelman Sciences, Ann Arbor, MI) and stored at 6–8°C prior to use. To incubation medium that had been pre-applied to the cells, aliquots of CD and CD-solubilized lipid solutions were added to give the concentrations of CD, CH, and OA indicated in the figure legends and tables. Generally, there was a 40-fold dilution of the CD and CD-solubilized lipid solutions at the time of administration to the cells. The dilution step was immediately followed by incubation on a rocking platform at 37°C for a minimum of 60 min.

Pulse-labeling of HepG2 cells, collection of cells and medium, and immunoprecipitation of apoB and albumin. Following treatments described in the figure legends and tables, cells were washed twice with warm PBS and administered serum-free, leucine-free labeling medium containing 100 μCi [^{3}H] leucine per mL for 10 min. Labeling medium was removed, cells were washed twice with warm PBS, and chased with serum-free medium for either 10 min to measure [^{3}H] apoB and [^{3}H] albumin synthesis or for as much as 190 min to measure the cellular content of undegraded [^{3}H] apoB, as well as the secretion of [^{3}H] apoB and [^{3}H] albumin. Medium was collected into microtubes on crushed ice containing 59 μL (per mL of medium) of a protease inhibitor cocktail comprising 1 μL of 1 M benzamidine-HCl, 10 μL of 0.5 M Na_2-EDTA (pH 8.4), 10 μL of 1 M HEPES (pH 9), 3 μL of 287 mM phenylmethylsulfonyl fluoride (in ethanol), and 35 μL of aprotinin (100 kallikrien-inactivating units/mL). Cells were washed twice with cold PBS and collected with a plastic cell

scraper into microtubes with ice-cold pH 8 lysis buffer (generally 1 mL) comprised of: 50 mM Tris, 150 mM NaCl, 5 mM Na_2EDTA, 62.5 mM sucrose, 12.1 mM Na-deoxycholate, 0.5% Triton X-100, leupeptin (50 μg/mL), pepstatin A (50 μg/mL), and the protease inhibitor cocktail described above (59 μL/mL). Lysed cells were incubated on a rocking platform at 6–8°C overnight, and cell lysates were fractionated into a soluble cell extract and an insoluble cell pellet by centrifugation at 12,000 × *g* and 6–8°C for 20 min. An aliquot of each cell extract (generally 75 μL) was taken for quantification of soluble cell protein. Aliquots of cell extract and medium samples were taken for immunoprecipitation of apoB and albumin as previously described (54). In brief, aliquots of cell extracts and medium were combined with excess rabbit antihuman apoB antiserum, or with excess sheep antihuman albumin antiserum. ApoB and albumin were immunoprecipitated and eluted from protein A-sepharose CL-4B beads into electrophoresis sample buffer. An aliquot of each eluted immunoprecipitate was dispersed in 5 mL of Packard Ultima Gold liquid scintillation cocktail (Packard Instrument Co., Meriden, CT) and total [^{3}H] was counted on a Packard Tri-Carb 1500 liquid scintillation analyzer (Packard Instruments, Downers Grove, IL). Protein in a 15-μL aliquot of each eluted immunoprecipitate was separated by SDS-PAGE (3–15% gradient). Developed gels were incubated for 20 min at room temperature in Autofluor (National Diagnostics, Inc., Manville, NJ), dried, and exposed to Kodak X-OMAT XAR-2 film (Sigma catalog # F-5763). The [^{3}H] apoB and [^{3}H] albumin bands were quantified on an Ultroscan XL enhanced laser densitometer (Pharmacia LKB Biotechnology, Bromma, Sweden). Total [^{3}H] apoB and [^{3}H] albumin per sample were calculated from the percentage of total immunoprecipitable [^{3}H] that was present in the [^{3}H] apoB or [^{3}H] albumin band of each autoradiograph.

Incorporation of [^{14}C] acetate into CH. For the experiment depicted in Tables 3 and 4, cells were chased with 2.5 mL of serum-free medium containing 5 μCi [^{14}C] acetate. At the termination of the chase period, medium and cells were collected as described above. Aliquots (1 mL) of medium and cell extracts were frozen at −80°C and lyophilized to reduce the aqueous volume. Medium, cell extract, and cell pellet samples were suspended in 200 μL of H_2O, and lipids were extracted by the method of Folch (55) as modified by Bligh and Dyer (56). Washed extracts were evaporated to dryness under N_2 and redissolved in 200 μL $CHCl_3$/MeOH (2:1, vol/vol). Aliquots (50 μL) of each lipid extract were spotted onto 20 cm × 20 cm silica gel 60 thin-layer chromatography plastic-backed plates (E. Merck, Darmstadt, Germany). A CH/cholesteryl OA standard was spotted in each sample lane, as well as in two independent lanes per plate. Chromatograms were developed with hexane/diethyl ether/glacial acetic acid (80:20:1, by vol). CH and cholesteryl ester were visualized with I_2 vapor, and the silica gel containing the bands was scraped with a razor blade into scintillation vials. Samples were dispersed in 5 mL of scintillation cocktail, and the incorporation of [^{14}C] acetate into CH and total cholesteryl ester was quantified by liquid scintillation counting.

Analysis of CH, total cholesteryl ester, and total triglyceride mass. In the experiment depicted in Figure 4, cells for lipid analysis were treated in parallel to those treated for pulse-labeling and immunoprecipitation of apoB. The cells were collected in a lysis buffer as previously described, and a 75-μL aliquot of each cell lysate was taken for protein quantification. Neutral lipids in the remainder of each cell lysate were extracted by the method of Folch (55) as modified by Bligh and Dyer (56). Extracts were dried under N_2 and re-extracted with three 500-μL volumes of hexane. Hexane extracts were dried under N_2 and redissolved in 500 μL of isooctane/ tetrahydrofuran (99:1). Samples (20 μL) were loaded onto a 5-μm silica column (4.6 mm diameter × 10 cm; Phase Separations, Norwalk, CT; cat. #S5W) attached to a Varian 9012 high-pressure liquid chromatograph (Varian Associates, Inc., Walnut Creek, CA). CH, total cholesteryl ester (as cholesteryl OA), and total triglyceride (as triolein) bands were resolved and eluted with a three-solvent gradient (isooctane:tetrahydrofuran/acetone:methylene-Cl/isopropanol:H_2O), and measured with an evaporative light-scattering detector (Sedere #Sedex 55; Richard Scientific, Novato, CA). Lipid mass was quantified from external standard curves.

Cell protein and [^{3}H] protein analysis. Aliquots of cell extracts (75 μL) or medium (150 μL) were combined with 15 μL of Na cholate solution (10 mg/mL) and 15 μL of 100% trichloroacetic acid per 75 μL aliquot. Samples were mixed, and protein was precipitated at 6–8°C for 60 min. Samples were centrifuged at 10,000 × *g* for 5 min, and pellets were washed with 500 μL of 12% trichloroacetic acid. Samples were recentrifuged and washed pellets were dissolved in 30 μL of 0.7 N NaOH plus 20 μL of 10% Na dodecylsulfate. The sample volume was brought up to 500 μL with a warm (pH 7.2) 50-mM KH_2PO_4 solution. Aliquots were taken for cell protein quantification by the method of Lowry (57), using BSA as a standard. Immunoprecipitable [^{3}H] protein was measured by liquid scintillation analysis as previously described.

Statistical analysis. Data were analyzed on SAS, using a one-way analysis of variance. Statistical significance for each model was set at $P < 0.05$. Treatment means were separated with Fisher's LSD test ($P < 0.05$).

RESULTS

Administration of CD-solubilized OA. Earlier pulse-chase studies in HepG2 cells showed that short-term treatment with 0.8 mM OA (complexed to 1.5% BSA) stimulated apoB intracellular transport, decreased apoB proteolysis, and increased apoB secretion (54). Other studies have demonstrated a BSA-dependent reduction in apoB synthesis (58) and secretion (59) that may confound the effects of OA. In the experiment depicted in Table 1, monolayers of HepG2 cells were pretreated for 1 h, as well as during the 2-h pulse-chase period, with either serum-free medium (Control), serum-free

TABLE 1
CD-Solubilized Oleate (OA) Rapidly Suppresses the Intracellular Degradation of Nascent ApoB[a]

		[^{3}H] ApoB				[^{3}H] Albumin	
Treatment	Cell protein (mg/well)	Synthesis [dpm ($\times 10^{-5}$)/ mg cell protein]	Undegraded in cell (% of synthesis)	Secreted (% of synthesis)	Cellular degradation (% of synthesis)	Synthesis [dpm ($\times 10^{-5}$)/ mg cell protein)	Secreted (% of synthesis)
Control	0.57 ± 0.15	7.1 ± 1.2^b	15.5 ± 1.4^c	4.5 ± 1.0^c	80.0 ± 1.1^b	27.9 ± 5.8	74.3 ± 4.4^c
CD	0.70 ± 0.02	6.4 ± 0.7^{b,c}	15.3 ± 0.6^c	4.2 ± 0.1^c	80.5 ± 0.5^b	29.3 ± 0.5	82.5 ± 5.9^c
OA + CD	0.60 ± 0.11	5.2 ± 0.7^c	64.3 ± 17.3^b	31.7 ± 19.9^b	4.0 ± 6.0^c	25.7 ± 13.8	98.6 ± 13.6^b

[a]HepG2 cells (70% confluent) were treated for 1 h in 1 mL of serum-free medium (SFM) containing one of the following additions: none (control), 1.5 mg (0.15% wt/vol) cyclodextrin (CD), or 61 μg Na oleate (0.2 mM) solubilized with 0.15% CD (OA + CD). All cells were pulsed for 10 min with 100 μCi [^{3}H] leucine in 1 mL of serum-free, leucine-free medium containing the additions described above. Cells from each treatment were chased in 1 mL of SFM (containing additions as above) for either 10 min to measure apoB and albumin synthesis or 100 min to measure the cellular content of undegraded apolipoprotein B-100 (apoB), as well as the secretion of nascent apoB and albumin. Cellular degradation of nascent apoB was calculated as the difference between apoB synthesis and the sum of cellular undegraded apoB and apoB secreted to the medium. Protocols are further described in the Materials and Methods section. Number of wells (*n*) each treatment: Control (*n* = 12), CD (*n* = 6), OA + CD (*n* = 12).
[b,c]Each value represents the mean ± SD. Values down a column with different letter superscripts are statistically different from each other at *P* (model) < 0.05.

medium containing 0.15% (wt/vol) CD (CD), or serum-free medium containing 0.2 mM OA solubilized with 0.15% CD (OA + CD). Albumin synthesis and secretion in this and other experiments were measured as a general control for protein synthesis and secretion. The data show that 0.15% CD alone had no effect on apoB or albumin synthesis and secretion. Administration of CD-solubilized OA modestly reduced apoB synthesis. However, the cellular content of undegraded apoB and apoB secretion was increased 4-fold and 7-fold, respectively. Albumin synthesis was not affected by CD-solubilized OA, although albumin secretion was modestly increased by 24%. The marked reduction in total apoB degradation exceeded that previously reported in HepG2 cells when 1.5% BSA was used to solubilize 0.8 mM OA (54). These results demonstrate the utility of CD as a vehicle for the delivery of OA to cells in tissue culture.

Independent effects of CD on apoB proteolysis and secretion. In the experiment depicted in Table 2, HepG2 cells were incubated for 4 d with 2 mL of complete growth medium (+10% FBS) containing either 0 (control), 0.5, 1, or 1.5% (wt/vol) CD. Medium was removed and fresh medium was applied every 24 h. CD treatment had no effect on either apoB or albumin synthesis. However, secretion of nascent apoB was nearly doubled as the CD concentration was increased from 0 to 0.5%. Although, there was no further significant increase in apoB secretion at higher CD concentrations, there was a CD dose-dependent increase in the cellular content of undegraded apoB.

Although hepatic CH synthesis has been suggested as a modulator of apoB-lipoprotein secretion *in vivo* (60), this has not been confirmed in most studies using cultured cells. In the experiment depicted in Table 3 and Table 4, the effects of both 1- and 4-d CD treatment on apoB production and the effect of CD treatment on the incorporation of [^{14}C] acetate into CH and cholesteryl ester were measured. Table 3 shows that secretion of nascent apoB was increased 2.4-fold in the CD (4-d) group, but only by 82% in the CD (1-d) group. The concentration of newly synthesized apoB remaining undegraded in cells was increased by an average of 20% in both CD-treated groups. Although the increase was not statistically significant in this particular experiment, a similar increase following treatment with 0.5% CD was consistently observed in

TABLE 2
Four-Day CD Treatment Increases the Cellular Content of Undegraded ApoB and Stimulates ApoB Secretion[a]

		[^{3}H] ApoB				[^{3}H] Albumin	
Treatment	Cell protein (mg/well)	Synthesis [dpm ($\times 10^{-5}$)/ mg cell protein]	Undegraded in cell (% of synthesis)	Secreted (% of synthesis)	Cellular degradation (% of synthesis)	Synthesis [dpm ($\times 10^{-5}$)/ mg cell protein)	Secreted (% of synthesis)
Control	1.24 ± 0.06^b	2.8 ± 0.1	20.4 ± 1.0^e	14.5 ± 7.3^c	65.5 ± 6.2^b	20.1 ± 1.6	92.4 ± 5.9^c
0.5% CD	1.21 ± 0.02^b	3.0 ± 0.1	25.7 ± 1.4^d	27.7 ± 3.1^b	46.6 ± 4.2^c	20.1 ± 1.7	93.9 ± 2.2^c
1.0% CD	1.14 ± 0.07^c	2.8 ± 0.1	32.4 ± 2.6^c	31.2 ± 0.7^b	36.4 ± 2.7^d	18.6 ± 0.1	101.6 ± 2.0^b
1.5% CD	1.06 ± 0.04^d	2.8 ± 0.4	39.6 ± 1.0^b	31.3 ± 3.3^b	29.0 ± 4.2^d	19.7 ± 1.1	90.4 ± 2.6^c

[a]HepG2 cells were treated for 4 d in 2 mL of complete growth medium (+10% fetal bovine serum) containing either 0 mg (control), 10 mg (0.5% wt/vol CD), 20 mg (1.0% CD), or 30 mg (1.5% CD) CD. Fresh medium was administered every 24 h. After 4 d, cells (100% confluent) were further treated for 1 h in 1 mL of serum-free medium containing 0 (control), 0.5, 1.0, or 1.5% CD. All cells were pulsed for 10 min with 100 μCi [^{3}H] leucine in 1 mL of serum-free, leucine-free medium containing the additions described above. Triplicate wells of cells from each treatment were chased in 1 mL of serum-free medium (containing additions as above) for either 10 min to measure apoB and albumin synthesis or 100 min to measure the cellular content of undegraded apoB, as well as the secretion of nascent apoB and albumin. Cellular degradation of nascent apoB was calculated as described in Table 1. Protocols are further described in the Materials and Methods section. See Table 1 for abbreviations.
[b,c,d,e] Each value represents the mean ± SD (*n* = 3, *n* = 6 for cell protein). Values down a column with different letter superscripts are statistically different from each other at *P* (model) < 0.05.

TABLE 3
Secretion and Degradation of Nascent ApoB in 1-d and 4-d CD-Treated Cells[a]

		[^{3}H] ApoB				[^{3}H] Albumin	
Treatment	Cell protein (mg/dish)	Synthesis [dpm ($\times 10^{-5}$)/ mg cell protein]	Undegraded in cell (% of synthesis)	Secreted (% of synthesis)	Cellular degradation (% of synthesis)	Synthesis [dpm ($\times 10^{-5}$)/ mg cell protein]	Secreted (% of synthesis)
Control	2.27 ± 0.12	4.9 ± 0.7	21.2 ± 2.2	6.9 ± 1.1[c]	71.9 ± 3.2	22.0 ± 0.9	64.5 ± 8.7
CD (1-d)	2.57 ± 0.17	4.5 ± 0.4	25.8 ± 2.1	12.6 ± 2.1[b,c]	61.6 ± 3.9	18.7 ± 4.0	71.8 ± 5.4
CD (4-d)	2.19 ± 0.07	5.3 ± 0.4	24.7 ± 4.6	16.8 ± 4.6[b]	58.5 ± 9.2	22.6 ± 3.0	69.1 ± 9.3

[a]HepG2 cells in 60-mm diameter dishes were treated in 5 mL of complete growth medium (+10% fetal bovine serum) without (control) or with 0.5% (wt/vol) CD. In control and CD (4-d) incubations, 5 mL of fresh medium was administered to cells every 24 h over the 4-d period. In CD (1-d) incubations, 5 mL of fresh medium without CD was administered to cells every 24 h during the first 3 d. During the subsequent 24 h, cells were administered 5 mL of fresh medium containing 0.5% CD four times (at 0, 4, 8, and 12 h into the 24-h period). Thus, 5 mL of fresh medium containing 0.5% (3.5 mM) CD was applied four times to both CD-treated groups during either a 1-d or 4-d period. After 4 d, cells were further incubated for 1 h in 2.5 mL of serum-free medium either without (control) or with 0.5% CD (both CD groups). All cells were pulsed for 10 min with 250 μCi [^{3}H] leucine in 2.5 mL of serum-free, leucine-free medium without or with 0.5% CD. Triplicate dishes of cells from each treatment were chased in 2.5 mL of serum-free medium without or with 0.5% CD for either 10 min to measure apoB and albumin synthesis or 100 min to measure the cellular content of undegraded apoB, as well as the secretion of nascent apoB and albumin. Cellular degradation of nascent apoB was calculated as described in Table 1. In dishes of cells chased for 100 min, 5 μCi [^{14}C] acetate was administered with the chase medium to measure CH biosynthesis. These latter results are provided in Table 4. Protocols are further described in the Materials and Methods section. See Table 1 for abbreviations.

[b,c]Each value represents the mean ± SD (n = 3, n = 6 for cell protein). Values down a column with different letter superscripts are statistically different from each other at P (model) < 0.05.

other experiments. Table 4 shows that the incorporation of [^{14}C] acetate into CH was not elevated after 4 d of daily CD administration, and the incorporation of [^{14}C] acetate into CH was only slightly elevated after 1 d of multiple CD applications. This is consistent with a transient (over a 1–2 d period) CD-mediated increase in the incorporation of [^{14}C] acetate into CH that we have observed (Peluso, M.R., and Dixon, J.L., unpublished data). After both 1- and 4-d CD treatments, the incorporation of [^{14}C] acetate into cholesteryl ester was reduced (Table 4). Therefore, the CD-mediated increase in apoB secretion did not occur in parallel with an increase in CH synthesis from acetate or an increase in the esterification of newly synthesized CH. Cyclodextrin did increase movement of nascent CH to the medium (Table 4). The increase in [^{14}C] CH appearing in the medium could have resulted from efflux to CD and from increased incorporation into secreted apoB-lipoprotein.

Comparison of the effects of CD and CD-solubilized OA on apoB production. In Figure 1, HepG2 cells were either untreated in complete growth medium (C), treated with 0.5% (3.5 mM) CD for 4 d in complete growth medium (CD), or treated with 0.15 mM OA solubilized with 0.5% CD in serum-free medium for 3 h (OA). The gel data show that the reduction in apoB degradation by OA was due to a combination of a 3.6-fold increase in cellular undegraded apoB and a 2.8-fold increase in apoB secretion. In contrast, the reduction in apoB degradation by CD was primarily due to a 2.3-fold increase in apoB secretion. Undegraded apoB was increased by only 25% in CD-treated cells. These CD-mediated effects were similar to those shown in Table 2 and Table 3.

In Table 5, to test whether the effects of 4-d CD and short-term OA treatments on apoB secretion were additive, cells treated for 4 d in complete growth medium with 0.5% CD were further treated for 3 h with graded concentrations of CD-solubilized OA (0–0.2 mM). As previously demonstrated, CD reduced apoB proteolysis and increased apoB secretion. The administration of CD-solubilized OA further reduced apoB proteolysis, but apoB secretion was not further increased during 100 min of chase. The gel data provided in Figure 2 illustrate a 70% net increase in cellular undegraded apoB as the OA concentration was increased from 0 to 0.2 mM.

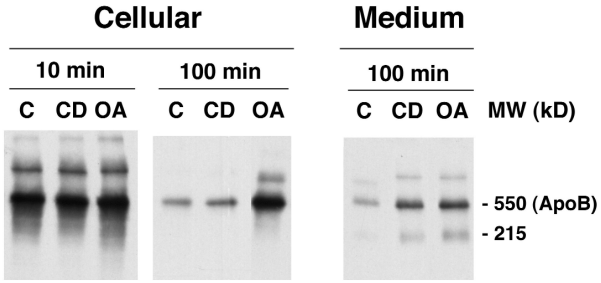

FIG. 1. Comparison of the effect of 3-h oleate (OA) and 4-d cyclodextrin (CD) treatments on apolipoprotein B-100 (apoB) production. HepG2 cells were cultured in 2 mL of complete growth medium (+10% fetal bovine serum). One group of cells was cultured for 4 d as a control (C). A second group was treated for 4 d with daily application of 0.5% (wt/vol) CD (CD). A third group was cultured for 4 d with complete medium and subsequently treated for 1 h prior to pulse-labeling with 1 mL of serum-free medium containing 0.15 mM oleate (OA) solubilized with 0.5% CD. The C and CD groups were similarly treated with 1 mL of serum-free medium without (C) or with 0.5% CD for 1 h prior to pulse-labeling. All cells were pulsed for 10 min with 100 μCi [^{3}H] leucine and triplicate wells from each group were chased for either 10 min or 100 min as previously described. ApoB was immunoprecipitated and quantified by scintillation counting, SDS-PAGE, and fluorography as described in the Materials and Methods section. The figure shows the gel data from one representative sample per treatment. In the C, CD, and OA groups, respectively: [^{3}H] apoB synthesis (cellular apoB at 10 min) averaged 6.2 ± 0.4 ($\times 10^5$), 6.3 ± 0.8 ($\times 10^5$), and 7.7 ± 0.4 ($\times 10^5$) dpm/mg cell protein; the cellular content of undegraded [^{3}H] apoB (cellular apoB at 100 min) averaged 1.1 ± 0.1 ($\times 10^5$), 1.4 ± 0.1 ($\times 10^5$), and 4.9 ± 0.6 ($\times 10^5$) dpm/mg cell protein; and [^{3}H] apoB secreted (medium apoB at 100 min) averaged 0.6 ± 0.2 ($\times 10^5$), 1.4 ± 0.2 ($\times 10^5$), and 2.1 ± 0.3 ($\times 10^5$) dpm/mg cell protein. Each value represents the mean ± SD (n = 3).

TABLE 4
Reduced Degradation of Nascent ApoB in CD-Treated Cells Is Not Accompanied by an Increase in Cholesterol Synthesis from [^{14}C] Acetate[a]

Treatment	[^{14}C] Cholesterol		[^{14}C] Cholesteryl ester	
	Cell	Medium	Cell	Medium
	[dpm ($\times 10^{-3}$)/mg cell protein]		[dpm ($\times 10^{-3}$)/mg cell protein]	
Control	77.2 ± 11.0[c]	3.0 ± 0.2[c]	5.93 ± 0.67[b]	0.28 ± 0.10[b]
CD (1-d)	94.9 ± 4.1[b]	8.3 ± 0.1[b]	2.72 ± 0.27[d]	0.13 ± 0.03[c]
CD (4-d)	85.8 ± 0.1[bc]	8.9 ± 0.6[b]	3.83 ± 0.04[c]	0.11 ± 0.01[c]

[a]HepG2 cells in 60-mm diameter dishes were treated and subjected to pulse-chase protocols as described in Table 3. The incorporation of [^{14}C] acetate (5 µCi/dish) into cellular CH and newly-synthesized CH appearing in the medium was measured over the 100 min chase period. Protocols are further described in the Materials and Methods section. See Table 1 for abbreviations.
[b,c,d]Each value represents the mean ± SD (n = 3). Values down a column with different letter superscripts are statistically different from each other at P (model) < 0.05.

Administration of CD-solubilized CH. The previous experiments have shown that CD can modestly reduce apoB proteolysis and markedly increase apoB secretion, possibly by modulating some aspect of CH metabolism related to CD-mediated CH efflux. To further examine a potential role for cellular CH in apoB-lipoprotein production, the effects of the administration of CD-solubilized CH with the culture medium were measured. In Table 6, HepG2 cells were treated for 4 d in complete growth medium either without (control) or with 0.5% (3.5 mM) CD. CD-treated cells were further treated for 3 h with graded concentrations of CD-solubilized CH (0–40 µg CH). Cyclodextrin-solubilized CH rapidly attenuated the increase in apoB secretion induced by 4-d CD treatment. Indeed, the administration of 20 µg CH decreased apoB secretion to one-third of that induced by CD. Higher doses of exogenous CH had no additional effect. With the application of CD-solubilized CH, albumin secretion was decreased by a maximum of only 20% . This latter result indicated that general inhibition of protein secretion was not the primary cause of the 67% reduction in apoB secretion with the administration of CD-solubilized CH.

In Figure 3, HepG2 cells were either untreated in complete growth medium (control), treated with 0.5% (3.5 mM) CD for 4 d, or treated with 7.5 µg/mL of CD-solubilized CH per d for 3 d followed by multiple applications of 0.5% CD alone for 1 d (CH + CD). Figure 3A shows that in the CD group, there was an increase in cellular undegraded apoB at 70 min, but not at 130 or 190 min of chase. This suggested a transient reduction in apoB proteolysis with CD treatment. Treatment with CD-solubilized CH followed by multiple applications of 0.5% CD alone increased cellular undegraded apoB at 70 and 130 min, but not at 190 min of chase. This suggested a more prolonged transient reduction in apoB proteolysis in cells pretreated with CH. Consistent with previous experiments, daily administration of 0.5% CD for 4 d markedly increased apoB secretion (Fig. 3B). ApoB secretion at 130 and 190 min of chase was 25% higher in CH-treated cells (CH + CD) than in cells treated with CD alone (CD). The results suggest that this specific pretreatment with CD-solubilized CH enhanced the CD-mediated increase in apoB production.

Prolonged treatment with CD and CD-solubilized CH: relationship of changes in cellular neutral lipid concentrations to apoB proteolysis and secretion. In Figure 4, Table 7, HepG2 cells were treated either with 0.5% CD throughout a 15-d period (0.5% CD group), or with CD at concentrations that included a progressive increase from 0.25 to 1% after cells were transferred into 6-well plates (0.25–1% CD group). The latter protocol was implemented to avoid a potentially toxic loss of CH from a small cell mass to a relatively high concentration of CD (1%). An additional group was treated with a constant concentration of CD-solubilized CH (20 µg/mL) for 14 d and subsequently for 1 d with 1% CD alone (CH group). The latter 1 d was simply an equilibration period that was not designed to greatly increase cellular CH efflux or modify cellular CH concentrations. Figure 4A shows that

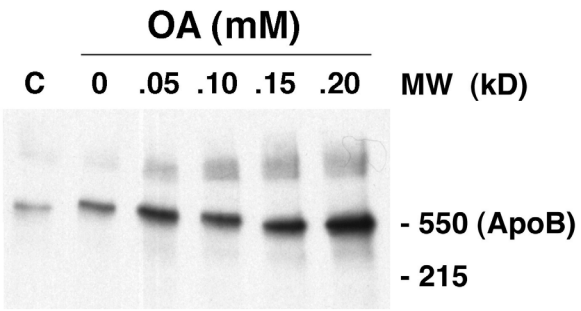

FIG. 2. OA causes a dose-dependent increase in cellular undegraded ApoB in 4-d CD-treated cells. HepG2 cells were treated and subjected to pulse-chase protocols as described in Table 5. ApoB was immunoprecipitated and quantified by scintillation counting, SDS-PAGE, and fluorography as described in the Materials and Methods section. The gel data from one representative sample per treatment is shown. There was a dose-responsive increase in the net cellular content of undegraded apoB at 100 min of chase as the OA concentration was increased from 0 to 0.2 mM. The net cellular content of undegraded [^{3}H] apoB averaged 1.2 ± 0.1 ($\times 10^5$), 1.7 ± 0.2 ($\times 10^5$), 2.2 ± 0.2 ($\times 10^5$), 2.1 ± 0.2 ($\times 10^5$), 2.5 ± 0.1 ($\times 10^5$), and 2.9 ± 0.5 ($\times 10^5$) dpm/mg cell protein in the control (C) and 0, 0.05, 0.10, 0.15, and 0.20 mM OA groups, respectively. Each value represents the mean ± SD (n = 3). See Figure 1 for abbreviations.

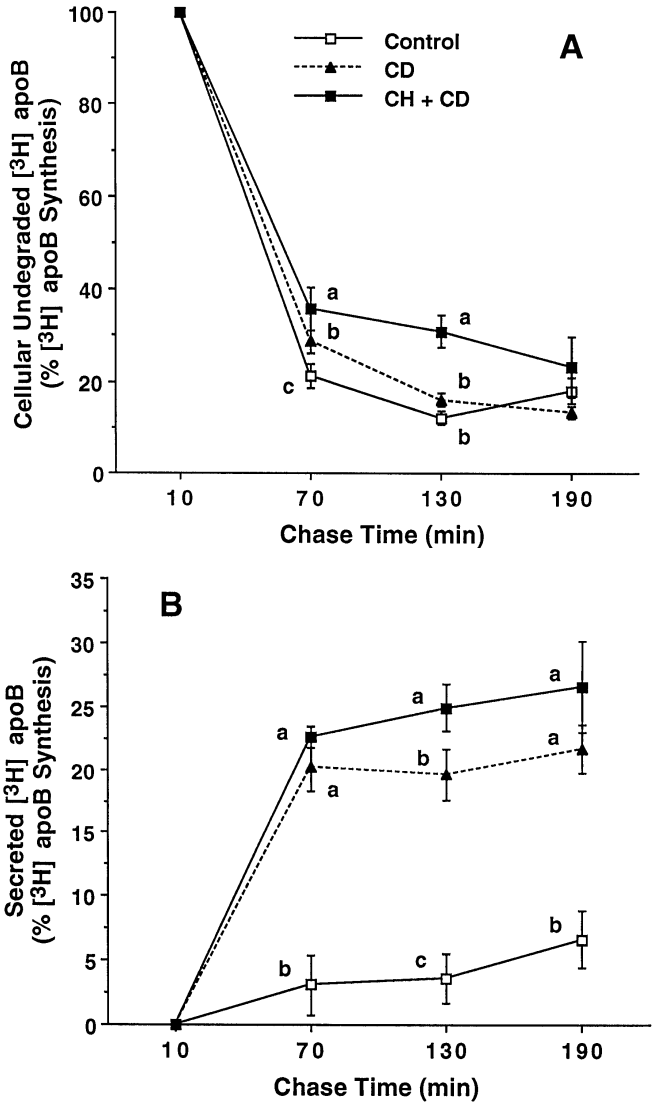

FIG. 3. Three-day pretreatment with CD-solubilized CH, followed by 1 d of multiple CD applications suppresses apoB proteolysis and increases apoB secretion. HepG2 cells were treated for 3 d in 2 mL complete growth medium (+10% fetal bovine serum) containing one of the following additions: none (control), 10 mg (0.5% wt/vol) CD (CD), or 15 µg CH solubilized with 0.5% CD (CH + CD). Fresh medium was administered every 24 h. Cells were further incubated for 20 h in 2 mL complete medium without (control) or with 0.5% CD (CD and CH + CD groups), plus an additional 4 h in 1 mL serum-free medium without or with 0.5% CD. To increase cellular CH efflux in both CD-treated groups during this latter 4 h, fresh medium was administered three times at 1-h intervals beginning at the onset of the period. All cells (70% confluent) were pulsed for 10 min with 100 µCi [^{3}H] leucine in 1 mL serum-free, leucine-free medium without or with 0.5% CD. Triplicate wells of cells from each treatment group were chased in 1 mL serum-free medium without or with 0.5% CD for either 10 min to measure apoB synthesis or 70, 130, or 190 min to measure the cellular content of undegraded apoB (Panel A) and secreted apoB (Panel B). Protocols are further described in the Materials and Methods section. The synthesis of [^{3}H] apoB averaged 7.6 ± 1.0 $(\times 10^5)^b$, 10.2 ± 0.4 $(\times 10^5)^a$, and 8.2 ± 0.2 $(\times 10^5)^b$ dpm/mg cell protein in the control, CD, and CH + CD groups, respectively. Each data point represents the mean ± SD (n = 3). ^{a,b,c}At each time point during the chase period, data points with different letter denotations are statistically different from each other at P (model) < 0.05. See Figure 1 for other abbreviations.

the cellular free CH and cholesteryl ester concentrations were 76% and 10-fold higher, respectively, in CH-treated cells than in control cells. Interestingly, the cellular free CH concentration was 25 and 41% higher in the 0.5% CD and 0.25–1% CD groups, respectively, than in the control group. This occurred without an effect of CD on the cellular cholesteryl ester concentration. The cellular triglyceride concentration was an average of 59% lower after 15 d of CD treatment. Fourteen-day treatment with 20 µg CH/mL (followed by 1-d equilibration) had no effect on apoB or cell protein synthesis (see Fig. legend), proteolysis, or secretion (Fig. 4B and 4C). Following 15-d CD treatment, [^{3}H] apoB and [^{3}H] protein synthesis (per mg cell protein) were increased by an average of 32 and 11%, respectively. Similarly, the concentrations of [^{3}H] apoB and [^{3}H] protein remaining undegraded in cells at the end of the 90-min chase period were increased by an average of 34 and 17%, respectively. In contrast, there was a 2.8-fold and 3.7-fold increase in [^{3}H] apoB secretion in the 0.5% CD and 0.25–1% CD groups, respectively (Fig. 4B). Indeed, the relative percentage of newly synthesized apoB that was secreted increased from 8% in the control group to 18% in the 0.5% CD group to 23% in the 0.25–1% CD group. The results suggest that prolonged chronic CD treatment modestly increased the cellular free CH concentration, markedly increased the assembly of cellular triglyceride with nascent apoB, and markedly increased transport of apoB through the secretory pathway.

DISCUSSION

The effects of CD, CD-solubilized OA, and CD-solubilized CH on apoB production were measured in HepG2 cells. The experiments were designed to alter cellular CH metabolism, modify apoB secretion, suggest a discrete role for cellular CH in apoB-lipoprotein production, and compare the apparent effects of CH to the more established effects of triglyceride. Prolonged chronic treatment with CD was found to decrease intracellular apoB proteolysis and increase apoB secretion. The reduction in apoB proteolysis was proportional to the concentration of CD applied with the incubation medium from 0–1.5% (0–10.5 mM) CD. ApoB secretion approached the maximum (25–30% of apoB synthesized) with 0.5% (3.5 mM) CD.

The utilization of CD-solubilized OA provided insight to the competitive mechanisms regulating apoB proteolysis and secretion. For example, OA was a more potent inhibitor of rapid apoB proteolysis than CD. It has been suggested that OA rescues nascent apoB from rapid proteolysis and increases apoB secretion mainly through an increase in triglyceride synthesis (22,23,34,61). The observations that CD-solubilized OA reduced apoB proteolysis from near 70% (control) to less than 10% of apoB synthesized, and that apoB secretion during a 100-min chase period never exceeded 32% of apoB synthesized, indicated that a process other than inhibition of rapid apoB proteolysis was required for efficient apoB-lipoprotein secretion. Previous studies have shown that

TABLE 5
The Cellular Content of Undegraded ApoB in 4-D CD-Treated Cells Is Rapidly Enhanced by CD-Solubilized Oleate[a]

		[^{3}H] ApoB				[^{3}H] Albumin	
Treatment	Cell protein (mg/well)	Synthesis [dpm ($\times 10^{-5}$)/ mg cell protein]	Undegraded in cell (% of synthesis)	Secreted (% of synthesis)	Cellular degradation (% of synthesis)	Synthesis [dpm ($\times 10^{-5}$)/ mg cell protein]	Secreted (% of synthesis)
Control	0.85 ± 0.06	6.7 ± 0.7	17.6 ± 3.3^d	7.5 ± 1.1^d	74.8 ± 2.8^b	20.7 ± 2.7	118.2 ± 22.7^b
4-d CD plus 3-h OA:							
0 mM OA	0.84 ± 0.04	5.4 ± 0.5	31.6 ± 5.1^c	27.2 ± 3.3^{b,c}	41.2 ± 7.7^c	22.8 ± 0.9	101.2 ± 13.6^{bc}
0.05 mM OA	0.78 ± 0.05	6.1 ± 1.1	37.7 ± 11.2^c	25.3 ± 8.3^{b,c}	37.0 ± 19.5^c	21.9 ± 1.4	106.6 ± 7.2^{bc}
0.10 mM OA	0.85 ± 0.03	6.5 ± 0.8	32.8 ± 2.1^c	19.9 ± 1.6^c	47.3 ± 3.7^c	22.3 ± 0.8	92.8 ± 2.0^c
0.15 mM OA	0.86 ± 0.04	6.1 ± 0.4	41.6 ± 4.8^{b,c}	23.3 ± 3.1^{b,c}	35.1 ± 7.8^c	21.4 ± 2.1	93.0 ± 15.0^c
0.20 mM OA	0.86 ± 0.03	5.6 ± 0.1	52.2 ± 8.4^b	30.6 ± 1.4^b	17.2 ± 9.4^d	19.0 ± 1.1	88.3 ± 1.5^c

[a]HepG2 cells were treated for 4 d in 2 mL of complete growth medium (+10% fetal bovine serum) without (control) or with 0.5% (wt/vol) CD. Fresh medium was administered every 24 h. Cells (90% confluent) were further treated for 1 h in 1 mL of serum-free medium without (control) or with 0.5% CD. The incubation medium for the CD groups contained either 0, 0.05, 0.10, 0.15, or 0.20 mM oleate (OA) solubilized with 0.5% CD. All cells were pulsed for 10 min with 100 μCi [^{3}H] leucine in 1 mL of serum-free, leucine-free medium containing the same additions described for the 1-h treatment period. Triplicate wells of cells from each treatment were chased in 1 mL of serum-free medium (containing additions as above) for either 10 min to measure apoB and albumin synthesis or 100 min to measure the cellular content of undegraded apoB, as well as the secretion of nascent apoB and albumin. Cellular degradation of nascent apoB was calculated as described in Table 1. Protocols are further described in the Materials and Methods section. See Table 1 for abbreviations.
[b,c,d]Each value represents the mean ± SD (n = 3, n = 6 for cell protein). Values down a column with different letter superscripts are statistically different from each other at P (model) < 0.05.

small HDL-sized apoB-lipoprotein particles can be retained by HepG2 cells (62), and that sufficient lipid incorporation into nascent apoB at the ER is required for the formation of mature lipoprotein particles that are secreted (19). Rapid uptake of CD-solubilized OA and subsequent incorporation of the fatty acid into triglyceride near the site of apoB synthesis could have induced a large percentage of nascent apoB molecules to form into HDL-sized progenitor apoB-lipoprotein particles that were neither secreted nor degraded during the 100-min chase period. The results support the hypothesis that HepG2 cells have a limited capacity to complex triglyceride with small apoB-lipoprotein particles at the second step in the secretory pathway (17,63).

Triglyceride-facilitated transport of nascent apoB in the secretory pathway has been suggested to rescue apoB from proteolysis during OA administration (34). The mechanism by which prolonged CD treatment decreased apoB proteolysis and increased apoB secretion may have been different. *In vivo*, an increase in hepatic CH synthesis has been associated with increased VLDL production (60). However, CH synthesis was not increased when the CD-mediated decrease in apoB proteolysis and increase in apoB secretion were measured. Figure 4 showed that a marked increase in the cellular free and esterified CH concentrations after a 2-wk treatment with CD-solubilized CH did not affect apoB proteolysis or secretion. In 15-d CD-treated cells, a 25–41% increase in the

TABLE 6
The CD-Mediated Increase in ApoB Secretion Is Rapidly Reversed During Influx of CD-Solubilized Cholesterol[a]

		[^{3}H] ApoB				[^{3}H] Albumin	
Treatment	Cell protein (mg/well)	Synthesis [dpm ($\times 10^{-5}$)/ mg cell protein]	Undegraded in cell (% of synthesis)	Secreted (% of synthesis)	Cellular degradation (% of synthesis)	Synthesis [dpm ($\times 10^{-5}$)/ mg cell protein]	Secreted (% of synthesis)
Control	0.85 ± 0.06	7.0 ± 0.2	20.0 ± 1.6	2.2 ± 0.2^e	77.8 ± 1.4^b	21.2 ± 1.4^b	92.8 ± 8.1^b
4-d CD plus 3-h CH:							
0 μg CH	0.84 ± 0.04	7.6 ± 1.1	25.6 ± 3.4	18.9 ± 2.8^b	55.5 ± 6.2^d	20.1 ± 0.9^{b,c}	85.0 ± 2.4^{b,c}
10 μg CH	0.78 ± 0.05	6.8 ± 0.2	26.8 ± 2.7	10.7 ± 1.7^c	62.4 ± 4.4^{c,d}	18.2 ± 1.5^{c,d}	86.4 ± 9.9^{b,c}
20 μg CH	0.85 ± 0.03	6.5 ± 0.6	30.5 ± 7.8	5.9 ± 2.8^d	63.5 ± 10.6^{c,d}	17.6 ± 0.6^d	76.7 ± 14.1^{c,d}
30 μg CH	0.86 ± 0.04	7.4 ± 0.1	24.2 ± 2.6	5.5 ± 0.2^{d,e}	70.3 ± 2.8^{b,c}	16.2 ± 1.1^d	82.5 ± 2.2^{b,c,d}
40 μg CH	0.86 ± 0.03	6.6 ± 0.6	26.2 ± 2.4	5.2 ± 0.6^{d,e}	68.6 ± 3.1^{b,c}	17.1 ± 0.8^d	68.6 ± 2.6^d

[a]HepG2 cells were treated for 4 d in 2 mL of complete growth medium (+10% fetal bovine serum) without (control) or with 0.5% (wt/vol) CD. Fresh medium was administered every 24 h. Cells (90% confluent) were further treated for 1 h in 1 mL of serum-free medium without (control) or with 0.5% CD. The incubation medium for the CD groups contained either 0, 10, 20, 30, or 40 μg CH solubilized with 0.5% CD. All cells were pulsed for 10 min with 100 μCi [^{3}H] leucine in 1 mL of serum-free, leucine-free medium containing the same additions described for the 1-h treatment period. Triplicate wells of cells from each treatment were chased in 1 mL of serum-free medium (containing additions as above) for either 10 min to measure apoB and albumin synthesis or 100 min to measure the cellular content of undegraded apoB, as well as the secretion of nascent apoB and albumin. Cellular degradation of nascent apoB was calculated as described in Table 1. Protocols are further described in the Materials and Methods section. See Table 1 for abbreviations.
[b,c,d,e]Each value represents the mean ± SD (n = 3, n = 6 for cell protein). Values down a column with different letter superscripts are statistically different from each other at P (model) < 0.05.

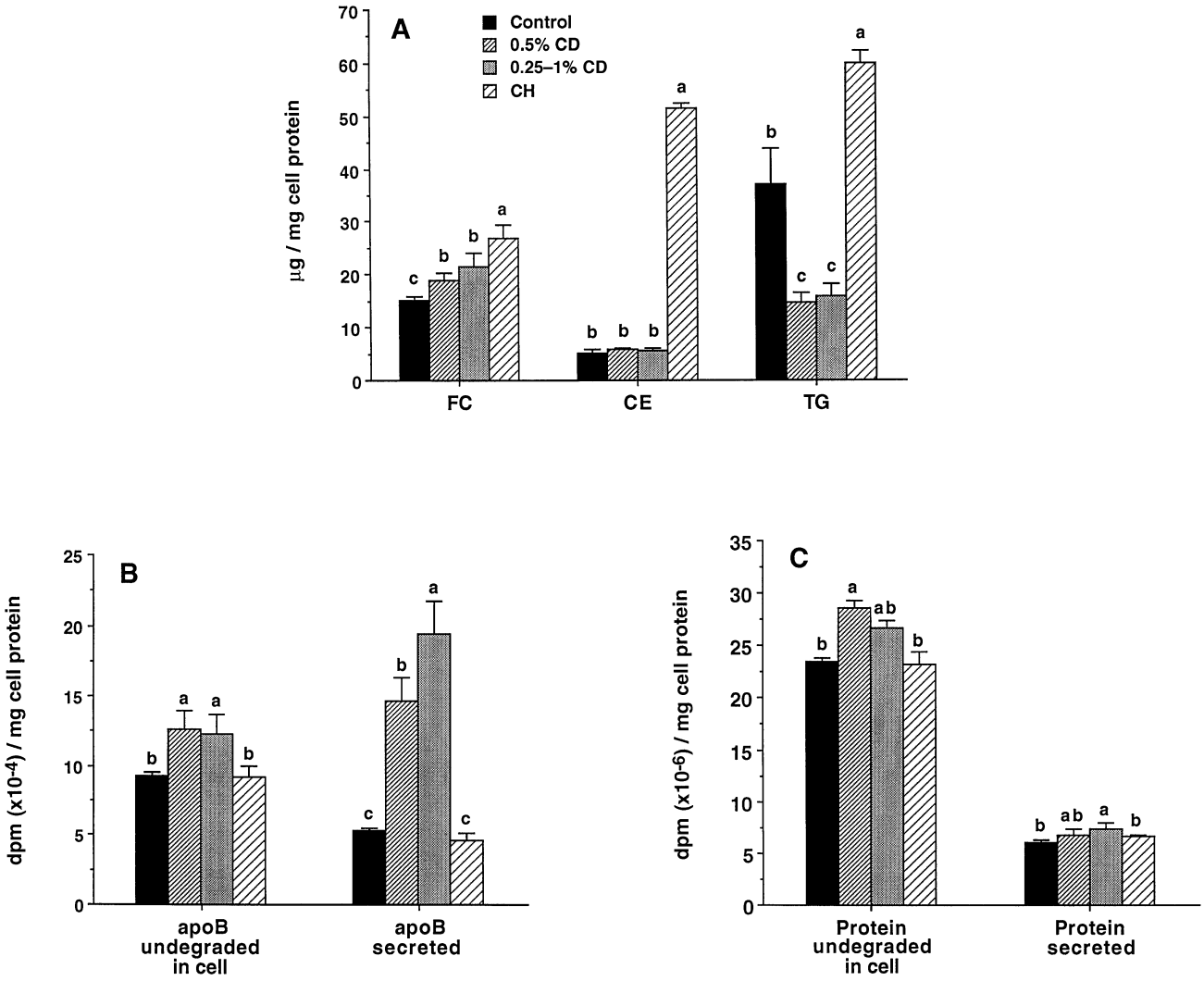

FIG. 4. Effect of 2-wk treatment with CD or CD-solubilized CH on cellular neutral lipid concentrations and apoB and cell protein degradation and secretion. HepG2 cells were cultured in 75-cm^2 flasks for 8 d with complete growth medium (+10% fetal bovine serum) (CM). During this period on d 1, 3, 5, and 7, cells were administered 10 mL CM plus either no addition (control), 0.5% (wt/vol) CD, or 200 µg CH complexed to 0.5% CD. On d 9, 4 separate groups of cells were seeded into 6-well tissue culture plates (9.6 cm^2/well, 1×10^5 cells/well) from the control (1 group), CD-treated (2 groups), and CH-treated (1 group) flasks. The four groups were further treated for 48 h (days 9–10) in 2 mL CM containing either no addition (control), 0.5% CD, 0.25% CD, or 40 µg CH (0.5% CD). Cells were cultured for five additional days (day 11–15) in 1.25 mL CM with the daily treatments indicated (see Table 7). Treatments were reapplied every 24 h (day 11–15). On d 16 (control cells 100% confluent), triplicate wells of cells from each group were collected for lipid analysis. Remaining cells were further treated as indicated for 1 h in 1 mL serum-free medium (SFM). Media were removed, and cells were pulsed for 10 min with 90 µCi [^{3}H] leucine in 1 mL serum-free, leucine-free medium (SFLFM) containing the indicated treatments. Triplicate wells of cells from each group were chased in 1 mL SFM containing the indicated treatments for either 10 min (to measure apoB and protein synthesis) or 90 min (to measure cellular undegraded and secreted apoB and protein). Cells were collected in lysis buffer, and cellular neutral lipids (Panel A), [^{3}H] apoB (Panel B), trichloroacetic acid (TCA)-precipitable [^{3}H] protein (Panel C), and TCA-precipitable cell protein were quantified as described in the Materials and Methods section. Cell protein averaged 1.19 ± 0.12^a, 0.91 ± 0.07^c, 0.88 ± 0.07^c, and 1.02 ± 0.04^b mg/well in the control, 0.5% CD, 0.25–1% CD, and CH groups, respectively ($n = 9$ each). Panel A: FC = free CH, CE = cholesteryl ester, TG = triglyceride. Panel B: [^{3}H] apoB synthesis averaged 62.6 ± 4.7 $(\times 10^4)^c$, 79.6 ± 12.3 $(\times 10^4)^{a,b}$, 86.4 ± 9.0 $(\times 10^4)^a$, and 66.3 ± 2.0 $(\times 10^4)^{b,c}$ dpm/mg cell protein in the control, 0.5% CD, 0.25–1.0% CD, and CH groups, respectively. Panel C: [^{3}H] protein synthesis averaged 34.5 ± 2.2 $(\times 10^6)^b$, 39.5 ± 2.7 $(\times 10^6)^a$, 37.1 ± 1.7 $(\times 10^6)^{a,b}$, and 35.7 ± 0.6 $(\times 10^6)^b$ dpm/mg cell protein in the control, 0.5% CD, 0.25–1.0% CD, and CH groups, respectively. Each bar in the figure represents the mean ± SD ($n = 3$). ^{a,b,c} Values with different letter denotations are statistically different from each other at P (model) < 0.05.

TABLE 7
Treatments Applied[a]

Treatment Group	days 9–10 (48 h)	day 11 (24 h)	day 12 (24 h)	day 13–14 (24 h × 2)	day 15 (24 h)	day 16 (1 h)	day 16 (10 min)	day 16 (10 or 90 min)
Control	CM	CM	CM	CM	CM	SFM	SFLFM	SFM
0.5% CD	0.5% CD	0.5% CD	0.5% CD	0.5% CD	0.5% CD	0.5% CD	0.5% CD	0.5% CD
0.25–1% CD	0.25% CD	0.5% CD	0.75% CD	1% CD	1% CD	1% CD	1% CD	1% CD
CH	40 μg CH	25 μg CH	25 μg CH	25 μg CH	1% CD	1% CD	1% CD	1% CD

[a]SFLFM, serum-free, leucine-free medium; CM, complete growth medium. See Table 1 for other abbreviations.

cellular free CH concentration was associated with a 25% reduction in apoB proteolysis, a 59% reduction in cellular triglyceride concentration, and a 3- to 4-fold increase in apoB secretion. Therefore, rather than a total cellular CH increase, we speculate that an increase in the CH concentration in discrete subcellular CH pools may have influenced apoB proteolysis and secretion in CD-treated HepG2 cells.

One possible mechanism responsible for the CD-mediated increase in apoB production may involve increased CH concentration in an apoB-associated ER regulatory pool. A putative ER regulatory pool of cholesterol modulates both LDL receptor activity and CH biosynthesis by controlling proteolytic release of sterol regulatory element-binding protein-1 (64). In the current studies, chronic and repeated CD-mediated CH efflux could have increased ER CH near the origin of and adjacent to the apoB secretory pathway, without greatly affecting the cellular total CH concentration. An increase in CH concentration in a specific regulatory pool could have "shielded" ER membrane-associated nascent apoB from rapid proteolysis, which is believed to be initiated by a putative ER protease (65).

A second possible mechanism for the CD-mediated increase in apoB production may involve increased CH transport through the putative ER regulatory pool to the plasma membrane. CD can stimulate efflux of newly-synthesized CH (Table 4), as well as CH from cells prelabeled with [^{3}H] CH (66,67). Although the pathway for CH trafficking from ER to plasma membrane diverges from that taken by secretory or integral membrane proteins, it is believed that both pathways have a common origin at the ER (68,69). We suggest that HepG2 cells adapted to chronic CD administration with increased CH flux through the nascent apoB-associated CH pool, which facilitated apoB-lipoprotein secretion. The concept of increased ER-to-plasma membrane CH flux in cells chronically treated with CD is supported by the reversal in CD-stimulated apoB secretion following the administration (and influx) of CD-solubilized cholesterol (Table 6). A CD-mediated increase in cellular LDL-CH uptake could also have contributed CH for increased flux through a regulatory (nascent apoB) pool. Indeed, LDL receptor activity has been shown to be elevated in HepG2 cells treated with 2.86 mM CD for 24 h (70). Increased LDL-cholesterol uptake would explain the observation that CH synthesis was not increased after 4 d of CD treatment in the presence of FBS (Table 4), despite daily removal of CD-solubilized cellular CH with each change of culture medium. Future studies will attempt to measure ER-to-plasma membrane cholesterol flux during chronic CD administration.

The administration of 3.5 mM CD for 4 d had minimal effects on cell culture growth. However, the application of higher CD concentrations for 4 d (Table 2) or repeated application of 3.5 mM CD for 15 d (Fig. 4) modestly attenuated culture growth. HepG2 cells have an absolute requirement for CH to maintain a normal growth rate (71). The administration of CD rapidly stimulates CH efflux, and cell protein synthesis may be transiently reduced when cells are exposed to a high dose of CD relative to the cell mass (Peluso, M.R., and Dixon, J.L., unpublished data).

In summary, treatment of HepG2 cells with CD-solubilized OA (0.2 mM) markedly reduced apoB proteolysis and markedly increased apoB secretion; thus, CD may be used instead of BSA to deliver exogenous OA to cultured cells. Chronic daily treatment of HepG2 cells with 0.5% (3.5 mM) CD modestly increased the cellular free CH concentration, modestly reduced apoB proteolysis, markedly reduced the cellular triglyceride concentration, and markedly increased apoB secretion. The administration of CD-solubilized CH increased the cellular free and esterified CH concentrations, but apoB production was not directly affected by the increase in cellular cholesterol. The use of CD as a research tool to modulate cellular CH metabolism may assist model formation to more clearly define mechanisms involved with disorders of hepatic CH metabolism linked with overproduction of apoB-lipoprotein.

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Capillary Supercritical Fluid Chromatography–Atmospheric Pressure Chemical Ionization Mass Spectrometry of γ- and α-Linolenic Acid Containing Triacylglycerols in Berry Oils

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ABSTRACT: The effect of the γ-linolenic acid (18:3n-6) residue on the elution of triacylglycerols on a 25% cyanopropyl-25% phenyl-50% methylpolysiloxane stationary phase was confirmed by using capillary supercritical fluid chromatography–atmospheric pressure chemical ionization mass spectrometry [cSFC–(APCI)MS]. The general elution rule on this stationary phase is that triacylglycerols having the same ACN + 2*n* value coeluted (ACN = acyl carbon number and *n* = combined number of double bonds in the acyl chains). The different effect of γ- and α-linolenic acid residues on the retention of triacylglycerols and the use of cSFC–(APCI)MS allowed the study of the number of different linolenic acid residue isomer combinations in triacylglycerols with an identical ACN and degree of unsaturation. Stearidonic acid (18:4n-3) residue was found to have a similar effect on the retention behavior of triacylglycerols as that of γ-linolenic acid residue. The abundance of the $[M - RCOO]^+$ ion, formed by the loss of one fatty acid moiety of a triacylglycerol, was found to be clearly higher in the case of γ-isomer of the linolenic acid than that of α-isomer in the identical regiospecific position. This indicates that the distance of the double bonds from the glycerol backbone in the acyl chain affects the stability of a triacylglycerol molecule in the (APCI)MS system. The triacylglycerol composition and the fatty acid combinations of triacylglycerols were found to be almost identical in black currant (*Ribes nigrum*) and alpine currant (*R. alpinum*) seed oils.

Lipids 32, 825–831 (1997).

The γ-isomer of linolenic acid (18:3n-6) should be regarded as an n-6 essential fatty acid having a notable role in health and disease as reviewed by Horrobin (1). Therefore, the analysis of triacylglycerols of edible oils containing γ-linolenic acid residues has a great importance and interest (2). Recently, the separation of triacylglycerols containing γ-linolenic and α-linolenic acid (18:3n-3) residues by using capillary supercritical fluid chromatography (cSFC) has been reported (3). The unique feature of cSFC in the separation of triacylglycerols on a 25% cyanopropyl-25% phenyl-50% methylpolysiloxane stationary phase is the order of the elution, which follows the rule of increasing ACN + 2*n* value, where ACN is the acyl carbon number and *n* is the combined number of double bonds in the acyl chains (4,5). The elution rule together with the effect of γ-linolenic acid residue on the retention resulted in an interesting elution pattern as the triacylglycerol containing the γ-isomer of linolenic acid eluted in a separate peak before that containing the α-isomer or no linolenic acid residues (3). The reported separation of the triacylglycerols of black currant and alpine currant seed oils provided, however, only limited information on the molecular species compositions, since a flame-ionization detector (FID) was used for detection.

The differentiation of linolenic acid isomers in mass spectrometric analysis of triacylglycerol mixtures requires chromatographic separation of the isomers, since both of them produce similar ions. Recently, a method for combining cSFC with atmospheric pressure chemical ionization mass spectrometry [(APCI)MS] for analyzing triacylglycerols in berry oils has been reported to achieve more detailed information about the molecular species (5). The method provided information on both the molecular weights and the possible fatty acid combinations of triacylglycerols. (APCI)MS also has been successfully utilized in combination with liquid chromatographic (LC) separation of triacylglycerols (6–9). Capillary SFC on a cyanopropyl stationary phase allows a different kind of approach for the analysis of triacylglycerols compared with LC, where the elution order in reversed-phase LC is according to increasing ACN – 2*n* values (10) or according to increasing degree of unsaturation, as in silver-ion LC (9,11). This may result in an advantage in the interpretation of cSFC–(APCI)MS spectra of triacylglycerols over those of LC–(APCI)MS mass spectra.

In this study, cSFC–(APCI)MS was applied to the analysis of triacylglycerols of black currant and alpine currant seed oils. Reliable information was achieved on the molecular weight species, as well as on the identities of the molecular associations of fatty acid residues in triacylglycerols. In addition, the ionization of linolenic acid residue isomers is briefly discussed.

EXPERIMENTAL PROCEDURES

Materials. The reference compounds 1,3-dioleoyl-2-γ-linolenoyl-*sn*-glycerol (*sn*-18:1n-9-18:3n-6-18:1n-9) and 1,3-dioleoyl-2-α-linolenoyl-*sn*-glycerol (*sn*-18:1n-9-18:3n-3-

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Abbreviations: ACN, acyl carbon number; APCI, atmospheric pressure chemical ionization; (APCI)MS, APCI mass spectrometry; cSFC, capillary supercritical fluid chromatography; FID, flame-ionization detector; LC, liquid chromatography.

18:1n-9) (custom-synthesized by Larodan, Malmö, Sweden) were diluted in *n*-hexane (Rathburn Chemicals Ltd., Walkerburn, Scotland) to a concentration of approximately 0.5 mg mL^{-1}. Black currant (*Ribes nigrum*) and alpine currant (*R. alpinum*) seed oil were extracted by supercritical carbon dioxide at Flavex (Rehlingen, Germany) in a production and a pilot plant, respectively. The oils were stored under nitrogen below 0°C, and diluted 10 mg in 1 mL of dichloromethane (Rathburn) before chromatography.

Methods. Supercritical fluid chromatographic separations were obtained on a Lee Scientific Series 600 supercritical fluid chromatograph (Dionex, Salt Lake City, UT) combined with a Finnigan MAT TSQ-700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) using an LC–APCI interface (Finnigan MAT). The interfacing method is described in detail elsewhere (5). A pneumatic and electrically controlled Valco switching valve (Valco Instruments, Inc., Houston, TX) with an internal loop size of 1.0 µL was used for timed/dynamic split injection. The timed split loop open time was 2.0 s. Two 10 m × 50 µm i.d. SB-Cyanopropyl-25 (25% cyanopropyl–25% phenyl–50% methylpolysiloxane; Dionex) columns combined in series with a zero dead volume butt connector (SGE, Austin, TX) were used for the chromatographic separations. Frit restrictors (30 cm × 50 µm i.d.; Dionex) were installed both after the analytical column and at the dynamic split outlet. Separations were performed at a constant temperature of 135°C with a linear density ramp from 0.16 g mL^{-1} at a rate of 0.010 g mL^{-1} min^{-1} to 0.62 g mL^{-1}. SFC-grade CO_2 (Scott Specialty Gases, Plumsteadville, PA) was used as a carrier fluid with a column flow rate of 0.18 mL min^{-1} measured with propane at the initial conditions of the chromatographic program with FID. The mass spectrometer was operated in a full scan mode using quadrupole 1 for scanning; quadrupoles 2 and 3 were operated in *rf*-only mode. Positively charged ions with *m/z* values 450–1000 were scanned with a scan time of 0.7 s. The corona needle current was set at 5.0 µA. Tuning of the mass spectrometer is described elsewhere (5). The APCI gases (N_2) during the cSFC–(APCI)MS operation were preheated by placing the Teflon gas-tube coils between the SFC oven outer wall and the oven cover. The sheath gas pressure was 1 bar, and the auxiliary gas flow rate was 5 mL min^{-1}. The APCI vaporizer temperature was kept at 425°C and the APCI capillary at 200°C. Methanol was used as a reactant ion solvent vapor, and introduced into the APCI source by leading the sheath gas flow through a 200-mL solvent bottle containing 50 mL of methanol (gradient grade; Merck, Darmstadt, Germany) so that the inlet tube was just beyond the solvent surface. The reactant ion solvent container was kept at ambient temperature.

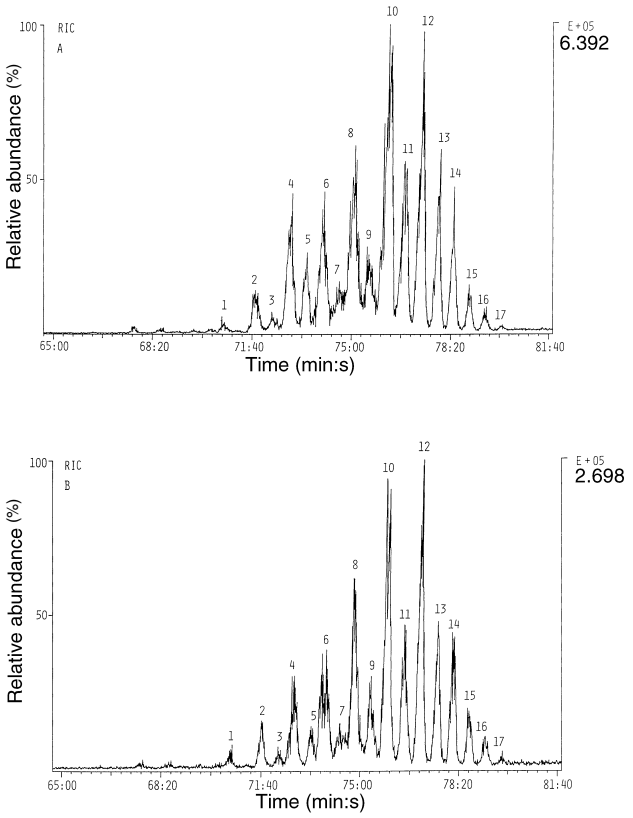

FIG. 1. The reconstructed ion chromatograms (RIC) of (A) black currant, and (B) alpine currant seed oil triacylglycerols obtained on capillary supercritical fluid chromatography–atmospheric pressure chemical ionization mass spectrometry (no smoothing was used in the chromatograms).

RESULTS AND DISCUSSION

The reconstructed cSFC–(APCI)MS ion chromatograms of black currant and alpine currant seed oil triacylglycerols separated on a 25% cyanopropyl-25% phenyl-50% methylpolysiloxane stationary phase are shown in Figure 1. The resolution was equal to or better than that on the same stationary phase reported earlier in the case of cSFC–FID (3). Methanol was used as a reactant ion solvent, which was previously shown to produce abundant $[M + H]^+$ and $[M - RCOO]^+$ ions from triacylglycerols (5). The $[M + H]^+$ ions provided the molecular weight information, whereas the $[M - RCOO]^+$ ions, formed by a loss of one fatty acid residue from a triacylglycerol, provided information on the possible fatty acid combinations of a molecule. Occasionally, $[M + 18]^+$ ions having low abundances were formed, but had no use in interpretation of the mass spectra in this study.

The effect of γ-linolenic acid residue on the elution of triacylglycerols on a 25% cyanopropyl–25% phenyl–50% methylpolysiloxane stationary phase in cSFC–FID has been demonstrated by a baseline separation of triacylglycerols *sn*-18:1n-9-18:3n-6-18:1n-9 and *sn*-18:1n-9-18:3n-3-18:1n-9 by Manninen *et al.* (3). The triacylglycerol containing the γ-linolenic acid residue eluted first, and the other containing the α-linolenic acid residue eluted with the same retention as those 54:5 triacylglycerols containing no linolenic acid residues. The detection with FID, however, could not provide information on the coeluting compounds. Therefore, no direct judgment could be made as to whether there were tri-

acylglycerols containing more than one γ-linolenic acid residue present in the studied oils or not.

The presence of isomeric triacylglycerols (equal number of acyl carbons and double bonds) was studied by plotting the cSFC–(APCI)MS ion chromatograms of the $[M + H]^+$ ions. For example, triacylglycerol 54:9 (*m/z* 873.8) eluted in four separate peaks (Fig. 2). Figure 3 shows, as an example, the mass spectra of the chromatographic peak numbers 14 and 15 in black currant seed oil. In addition, the mass spectral data extracted from peak numbers 13–16 are listed in Table 1. The mass spectra of triacylglycerols exhibited only abundant $[M + H]^+$ ions and $[M - RCOO]^+$ ions. The *m/z* values of the $[M + H]^+$ ions defined the molecular weight species of triacylglycerols present in the peaks: *m/z* 873.8 corresponded to the triacylglycerol 54:9, and *m/z* 875.8 corresponded to 54:8 in peak 14 (Fig. 3B). Peak 15 consisted of triacylglycerols 54:10 (*m/z* 871.9) and 54:9 (Fig. 3D). The presence of $[M - RCOO]^+$ ions in the mass spectra provided information on the possible fatty acid constituents of a triacylglycerol, as indicated in Figure 3A and C. Triacylglycerols eluting in the same chromatographic peak may yield the same fragment ions, which makes the interpretation of the spectra more complicated. The most probable fatty acid combination for triacylglycerol 54:9 in black currant seed oil is 18:3/18:3/18:3, where 18:3 is either α- or γ-linolenic acid moiety, according to the fatty acid analysis. In addition, fatty acid combinations 18:1/18:4/18:4 and 18:2/18:3/18:4 are also possible. The mass spectrum of each of the peaks 13–16 exhibited the fragment ion *m/z* 595.6 corresponding to the loss of one linolenic acid moiety from the molecule. Therefore, triacylglycerol 54:9 may represent the combination 18:3/18:3/18:3 in each of the four peaks. On the other hand, no loss of oleic acid from 54:9 (*m/z* 591.5) was found; thus, the combination 18:1/18:4/18:4 does not exist in black currant seed oil. In addition to the ion *m/z* 595.6, the mass spectra of peaks 14 and 15 provided ions corresponding to the loss of one linoleic acid (*m/z* 593.5) and one stearidonic acid (*m/z* 597.5) from the triacylglycerol 54:9 (Fig. 3). In peak 15,

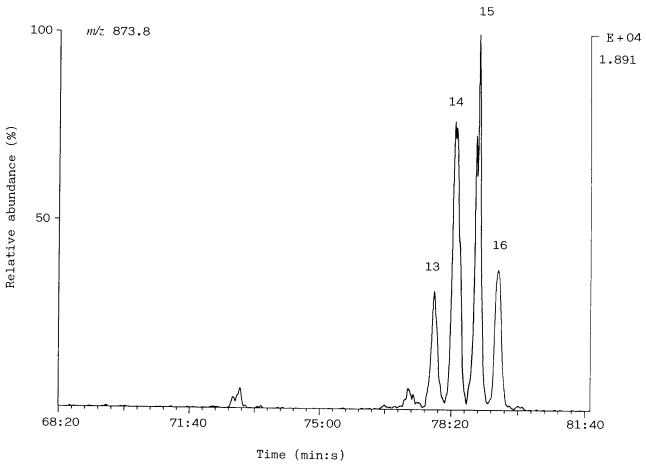

FIG. 2. The ion chromatogram of the molecular ion ($[M + H]^+$) of triacylglycerol 54:9 in black currant seed oil (7-point smoothing used in the ion chromatogram).

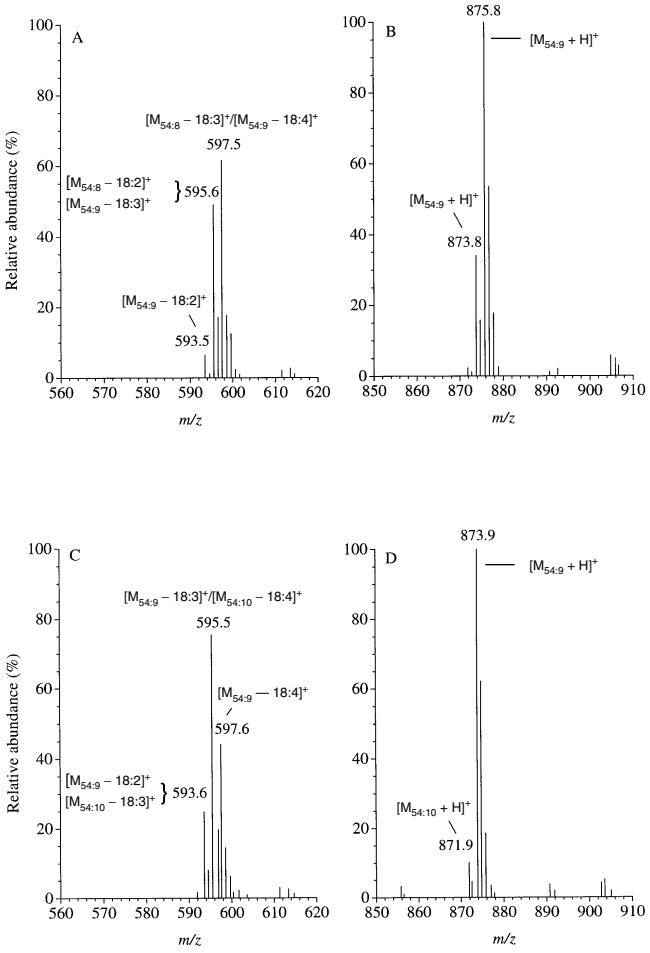

FIG. 3. The mass spectra of the chromatographic peak numbers 14 and 15 in black currant seed oil: (A) the $[M - RCOO]^+$-ion region of peak 14, (B) the $[M + H]^+$-ion region of peak 14, (C) the $[M - RCOO]^+$-ion region of peak 15, and (D) the $[M + H]^+$-ion region of peak 15. Peak numbers refer to those presented in Figure 1A and 2 and in Table 1.

the ion *m/z* 597.6 is produced only from 54:9 by the loss of a stearidonic acid. In addition, only a small portion of the abundances of ions *m/z* 593.6 and 595.5 were formed by the loss of linolenic acid and stearidonic acid from the triacylglycerol 54:10. According to these data, 54:9 in peak 15 is a mixture of 18:3/18:3/18:3 and 18:2/18:3/18:4. Peak 14 consists most probably of the same fatty acid combinations of 54:9, although a large portion of the ion abundances of *m/z* 595.6 and *m/z* 597.5 corresponded to the loss of fatty acid constituents from triacylglycerol 54:8. Tri-acylglycerol 54:9 in peak 16 yielded only one fragment ion (*m/z* 595.6) corresponding to the loss of linolenic acid moiety (Table 1). Furthermore, this peak was found to elute with the same retention time as 18:3n-3/18:3n-3/18:3n-3 in seed oils analyzed earlier (3–5). The molecular associations of fatty acid residues representing triacylglycerol 54:9 were judged based both on the mass spectral data and on the retention properties of triacylglycerols on the SB-Cyanopropyl column. In addition, comparison of the retention times and elution orders of triacylglycerols analyzed earlier with capillary SFC–(APCI)MS (5) with those of black currant

TABLE 1
Characteristic Ions of Triacylglycerols of Black Currant Seed Oil Obtained by Capillary Supercritical Fluid Chromatography–Atmospheric Pressure Chemical Ionization Mass Spectrometry, and Identification of the Molecular Species

Peak[a] number	$[M+H]^+$ *m/z* (%RA)[b]	Triacyl-glycerol	$[M-RCOO]^+$ *m/z* (%RA)[b] [ion]					Molecular structure[c]
1	859.8(1.9)	52:2	603.4(46.1)[M – 16:0]$^+$	577.5(100)[M – 18:1]$^+$				16:0/18:1/18:1
2	857.8(38.9)	52:3	601.7(100)[M – 16:0]$^+$	577.7(97.5)[M – 18:2]$^+$	575.7(60.4)[M – 18:1]$^+$			16:0/18:1/18:2
3	855.8(31.6)	52:4	599.6(37.2)[M – 16:0]$^+$	577.6(100)[M – 18:3]$^+$	573.6(16.7)[M – 18:1]$^+$			16:0/18:1/18:3n-6
4	855.7(100)	52:4	599.6(55.9)[M – 16:0]$^+$	577.5(11.5)[M – 18:3]$^+$	575.5(70.7)[M – 18:2]$^+$	573.6(8.2)[M – 18:1]$^+$		16:0/18:2/18:2, 16:0/18:1/18:3n-3
	885.8(5.0)	54:3	603.5(36.0)[M – 18:1]$^+$					18:1/18:1/18:1
5	853.8(100)	52:5	597.5(39.8)[M – 16:0]$^+$	577.6(19.9)[M – 18:4]$^+$	575.4(94.1)[M – 18:3]$^+$	573.7(18.1)[M – 18:2]$^+$	571.5(4.0)[M – 18:1]$^+$	16:0/18:2/18:3n-6, 16:0/18:1/18:4
6	853.8(75.3)	52:5	597.5(73.1)[M – 16:0]$^+$	575.6(35.3)[M – 18:3]$^+$	573.4(49.1)[M – 18:2]$^+$			16:0/18:2/18:3n-3
	851.7(15.2)	52:6	595.5(14.0)[M – 16:0]$^+$	573.5(49.1)[M – 18:3]$^+$				16:0/18:3n-6/18:3n-6
	883.8(62.8)	54:4	603.5(77.8)[M – 18:2]$^+$	601.5(100)[M – 18:1]$^+$	599.6(12.4)[M – 18:0]$^+$			18:1/18:1/18:2, 18:0/18:2/18:2
7	851.8(100)	52:6	595.5(58.7)[M – 16:0]$^+$	575.6(56.2)[M – 18:4]$^+$	573.6(39.8)[M – 18:3]$^+$	571.7(7.2)[M – 18:2]$^+$		16:0/18:3n-3/18:3n-6, 16:0/18:2/18:4
	881.8(43.8)	54:5	603.7(21.6)[M – 18:3]$^+$	601.5(5.9)[M – 18:2]$^+$	597.6(48.8)[M – 18:0]$^+$			18:0/18:2/18:3n-6
8	881.8(100)	54:5	601.6(93.7)[M – 18:2]$^+$	599.4(65.9)[M – 18:1]$^+$				18:1/18:2/18:2
9	879.8(100)	54:6	603.5(4.7)[M – 18:4]$^+$	601.7(48.8)[M – 18:3]$^+$	599.6(8.5)[M – 18:2]$^+$	597.6(17.4)[M – 18:1]$^+$		18:1/18:2/18:3n-6, 18:1/18:1/18:4
10	879.8(100)	54:6	601.6(17.6)[M – 18:3]$^+$	599.5(88.3)[M – 18:2]$^+$	597.5(22.7)[M – 18:1]$^+$			18:2/18:2/18:2, 18:1/18:2/18:3n-3
11	877.8(100)	54:7	601.6(10.0)[M – 18:4]$^+$	599.5(68.6)[M – 18:3]$^+$	597.5(55.9)[M – 18:2]$^+$	595.4(14.7)[M – 18:1]$^+$		18:2/18:2/18:3n-6, 18:1/18:3n-3/18:3n-6, 18:1/18:2/18:4
12	877.7(100)	54:7	599.6(29.0)[M – 18:3]$^+$	597.6(70.3)[M – 18:2]$^+$	595.6(11.8)[M – 18:1]$^+$			18:2/18:2/18:3n-3, 18:1/18:3n-3/18:3n-3
	875.8(16.3)	54:8	597.6(70.3)[M – 18:3]$^+$	595.6(11.8)[M – 18:2]$^+$				18:2/18:3n-6/18:3n-6
13	875.7(100)	54:8	599.5(14.8)[M – 18:4]$^+$	597.5(53.2)[M – 18:3]$^+$	595.5(31.3)[M – 18:2]$^+$	593.5(3.3)[M – 18:1]$^+$		18:2/18:3n-3/18:3n-6, 18:1/18:3n-3/18:4, 18:2/18:2/18:4
	873.8(7.3)	54:9	595.5(31.3)[M – 18:3]$^+$					18:3n-6/18:3n-6/18:3n-6
14	875.8(100)	54:8	597.5(61.6)[M – 18:3]$^+$	595.6(49.0)[M – 18:2]$^+$				18:2/18:3n-3/18:3n-3
	873.7(34.0)	54:9	597.5(61.6)[M – 18:4]$^+$	595.6(49.0)[M – 18:3]$^+$	593.5(6.4)[M – 18:2]$^+$			18:3n-3/18:3n-3/18:3n-6, 18:2/18:3n-6/18:4
15	873.9(100)	54:9	597.6(44.1)[M – 18:4]$^+$	595.5(75.4)[M – 18:3]$^+$	593.6(24.9)[M – 18:2]$^+$			18:3n-3/18:3n-3/18:3n-6, 18:2/18:3n-3/18:4
	871.9(10.2)	54:10	595.5(75.4)[M – 18:4]$^+$	593.6(24.9)[M – 18:3]$^+$				18:3n-6/18:3n-6/18:4
16	873.7(95.4)	54:9	595.6(100)[M – 18:3]$^+$					18:3n-3/18:3n-3/18:3n-3
	871.7(57.7)	54:10	595.6(100)[M – 18:4]$^+$	593.5(24.3)[M – 18:3]$^+$	591.4(3.9)[M – 18:2]$^+$			18:3n-3/18:3n-6/18:4, 18:2/18:4/18:4
17	871.6(100)	54:10	595.6(28.9)[M – 18:4]$^+$	593.4(48.7)[M – 18:3]$^+$				18:3n-3/18:3n-3/18:4

[a]Peak numbers refer to those in Figure 1A. [b]Relative abundance (RA) (%) of an ion in a normalized mass spectrum. [c]Molecular structure does not define the stereospecific position of fatty acids.

seed oil was useful. Thus, the most probable fatty acid combinations of the triacylglycerol 54:9 in the four peaks were: 18:3n-3/18:3n-3/18:3n-3 in peak 16, 18:3n-3/18:3n-3/18:3n-6 and 18:2/18:3n-3/18:4 in peak 15, 18:3n-3/18:3n-6/18:3n-6 and 18:2/18:3n-6/18:4 in peak 14, and 18:3n-6/18:3n-6/18:3n-6 in peak 13. The effect of ^{13}C and ^{18}O isotopes has to be taken into account in the interpretation of the mass spectra; the abundance of the fragment having a given *m/z* value will be slightly overstated owing to contributions of the molecule two *m/z* units lower containing two ^{13}C isotopes or one ^{18}O isotope.

Black currant and alpine currant seed oil contained small proportions of stearidonic acid (18:4n-3), approximately 4.4 and 3.2%, respectively (3). No ions corresponding to the loss of stearidonic acid were found in any of the mass spectra of the chromatographic peaks representing triacylglycerols containing only the α-isomer of linolenic acid or no linolenic acid residues (Table 1). However, stearidonic acid residue was found, for example, in the peak representing triacylglycerol 54:6 (peak number 9 in Table 1), having the possible fatty acid compositions 18:1/18:2/18:3n-6 and 18:1/18:1/18:4. In addition, triacylglycerol 54:10 as an example was found to be present in such a peak (peak number 15 in Table 1, Fig. 3C and D), which in principle should contain three γ-linolenic acid residues according to the retention order. However, the only possible fatty acid combination for triacylglycerol 54:10 in this peak was found to be 18:3n-6/18:3n-6/18:4. These kinds of observations led to the assumption that stearidonic acid residues similarly affected the retention of triacylglycerols as γ-linolenic acid residue did. The same phenomenon also has been found in silver-ion LC (9).

The fatty acid combinations of black currant and alpine currant seed oil triacylglycerols presented in Tables 1 and 2, respectively, were judged on the basis that γ-linolenic and stearidonic acid residues have a similar effect on the retention of triacylglycerols. The comparison of these two tables revealed that both seed oils are very much alike in the molecular composition as well as in the fatty acid combinations of the triacylglycerols. This study confirmed the tentative identification of the separated molecular species, by both the acyl carbon number and number of double bonds in the acyl chains, of these oils analyzed previously by cSFC–FID (3). In addition, the use of (APCI)MS for detection made it possible to identify coeluting components based on the presence of the $[M + H]^+$ ions in the mass spectra. Furthermore, (APCI)MS provided information on the fatty acid residues, which was not available with SFC–FID. Both black currant and alpine currant seed oil also contained small relative amounts of triacylglycerols with 56 acyl carbons. However, their fatty acid combinations could not be concluded in this study, since they coeluted with much more abundant triacylglycerols with 54 acyl carbons and, thus, produced too low an abundance of the fragment ions to be recognized with certainty. This was already seen in the earlier study of cloudberry and sea buckthorn seed oil (5).

The proportion of triacylglycerols in both of the oils was roughly estimated according to the relation of the abundances of $[M + H]^+$ ions of each triacylglycerol (Table 3). This al-

TABLE 2

Characteristic Ions of Triacylglycerols of Alpine Currant Seed Oil Obtained by Capillary Supercritical Fluid Chromatography–Atmospheric Pressure Chemical Ionization Mass Spectrometry, and Identification of the Molecular Species

Peak[a] number	$[M + H]^+$ *m/z* (%RA)[b]	Triacyl-glycerol	$[M - RCOO]^+$ *m/z* (%RA)[b] [ion]					Molecular structure[c]
1	859.8(2.4)	52:2	603.6(49.8)$[M - 16:0]^+$	577.6(100)$[M - 18:1]^+$				16:0/18:1/18:1
2	857.9(33.4)	52:3	601.5(100)$[M - 16:0]^+$	577.5(92.4)$[M - 18:2]^+$	575.5(39.8)$[M - 18:1]^+$			16:0/18:1/18:2
	887.6(1.3)	54:2	605.6(5.7)$[M - 18:1]^+$	603.6(8.6)$[M - 18:0]^+$				18:0/18:1/18:1
3	855.8(23.4)	52:4	599.7(41.3)$[M - 16:0]^+$	577.6(100)$[M - 18:3]^+$	573.6(12.3)$[M - 18:1]^+$			16:0/18:1/18:3n-6
4	855.8(66.0)	52:4	599.7(64.9)$[M - 16:0]^+$	577.6(12.9)$[M - 18:3]^+$	575.7(52.1)$[M - 18:2]^+$	573.5(6.9)$[M - 18:1]^+$		16:0/18:2/18:2, 16:0/18:1/18:3n-3
	885.9(5.0)	54:3	603.5(100)$[M - 18:1]^+$					18:1/18:1/18:1
5	853.8(100)	52:5	597.7(72.2)$[M - 16:0]^+$	577.6(41.8)$[M - 18:4]^+$	575.6(91.5)$[M - 18:3]^+$	573.6(22.2)$[M - 18:2]^+$	571.7(3.3)$[M - 18:1]^+$	16:0/18:2/18:3n-6, 16:0/18:1/18:4
	883.7(8.2)	54:4	605.6(3.0)$[M - 18:3]^+$	601.5(15.1)$[M - 18:1]^+$	599.5(1.6)$[M - 18:0]^+$			18:0/18:1/18:3n-6
6	853.8(32.3)	52:5	597.5(12.7)$[M - 16:0]^+$	575.7(9.7)$[M - 18:3]^+$	573.6(12.6)$[M - 18:2]^+$			16:0/18:2/18:3n-3
	851.7(5.1)	52:6	595.4(2.6)$[M - 16:0]^+$	573.6(12.6)$[M - 18:3]^+$				16:0/18:3n-6/18:3n-6
	883.8(41.7)	54:4	603.5(68.1)$[M - 18:2]^+$	601.5(100)$[M - 18:1]^+$	599.5(12.7)$[M - 18:0]^+$			18:1/18:1/18:2, 18:0/18:2/18:2
7	851.8(34.5)	52:6	595.5(25.7)$[M - 16:0]^+$	573.4(12.9)$[M - 18:3]^+$				16:0/18:3n-3/18:3n-6,
	881.8(24.9)	54:5	603.6(100)$[M - 18:3]^+$	599.6(45.1)$[M - 18:1]^+$				18:1/18:1/18:3n-6
8	881.8(100)	54:5	603.6(16.6)$[M - 18:3]^+$	601.6(79.4)$[M - 18:2]^+$	599.5(97.5)$[M - 18:1]^+$			18:1/18:1/18:3n-3, 18:1/18:2/18:2
9	879.7(100)	54:6	603.7(17.2)$[M - 18:4]^+$	601.4(69.8)$[M - 18:3]^+$	599.6(33.4)$[M - 18:2]^+$	597.6(46.6)$[M - 18:1]^+$		18:1/18:2/18:3n-6, 18:1/18:1/18:4
10	879.8(100)	54:6	601.6(25.0)$[M - 18:3]^+$	599.5(60.7)$[M - 18:2]^+$	597.6(24.0)$[M - 18:1]^+$			18:2/18:2/18:2, 18:1/18:2/18:3n-3
11	877.7(100)	54:7	601.7(18.8)$[M - 18:4]^+$	599.6(75.5)$[M - 18:3]^+$	597.6(62.1)$[M - 18:2]^+$	595.6(21.7)$[M - 18:1]^+$		18:2/18:2/18:3n-6, 18:1/18:3n-3/18:3n-6, 18:1/18:2/18:4
12	877.8(100)	54:7	599.5(37.1)$[M - 18:3]^+$	597.5(63.2)$[M - 18:2]^+$	595.5(13.9)$[M - 18:1]^+$			18:2/18:2/18:3n-3, 18:1/18:3n-3/18:3n-3
	875.7(15.1)	54:8	597.5(63.2)$[M - 18:3]^+$	595.5(13.9)$[M - 18:2]^+$				18:2/18:3n-6/18:3n-6
13	875.8(100)	54:8	599.5(39.8)$[M - 18:4]^+$	597.6(61.3)$[M - 18:3]^+$	595.6(33.0)$[M - 18:2]^+$	593.5(5.5)$[M - 18:1]^+$		18:2/18:3n-3/18:3n-6, 18:1/18:3n-3/18:4, 18:2/18:2/18:4
	873.8(6.0)	54:9	595.5(33.0)$[M - 18:3]^+$					18:3n-6/18:3n-6/18:3n-6
14	875.7(100)	54:8	597.6(56.6)$[M - 18:3]^+$	595.6(30.7)$[M - 18:2]^+$				18:2/18:3n-3/18:3n-3
	873.7(22.0)	54:9	597.6(56.6)$[M - 18:4]^+$	595.6(30.7)$[M - 18:3]^+$	593.4(5.5)$[M - 18:2]^+$			18:3n-3/18:3n-6/18:3n-6, 18:2/18:3n-6/18:4
15	873.7(100)	54:9	597.6(50.3)$[M - 18:4]^+$	595.5(55.5)$[M - 18:3]^+$	593.4(17.0)$[M - 18:2]^+$			18:3n-3/18:3n-3/18:3n-6, 18:2/18:3n-3/18:4
	871.8(8.4)	54:10	595.5(55.5)$[M - 18:4]^+$	593.4(17.0)$[M - 18:3]^+$				18:3n-6/18:3n-6/18:4
16	873.6(100)	54:9	595.5(99.1)$[M - 18:3]^+$					18:3n-3/18:3n-3/18:3n-3
	871.8(62.7)	54:10	595.5(99.1)$[M - 18:4]^+$	593.5(33.9)$[M - 18:3]^+$	591.4(2.8)$[M - 18:2]^+$			18:3n-3/18:3n-6/18:4, 18:2/18:4/18:4
17	871.6(100)	54:10	595.6(40.2)$[M - 18:4]^+$	593.4(39.4)$[M - 18:3]^+$				18:3n-3/18:3n-3/18:4

[a]Peak numbers refer to those in Figure 1B. [b]Relative abundance (RA) (%) of an ion in a normalized mass spectrum. [c]Molecular structure does not define the stereospecific position of fatty acids.

TABLE 3
The Proportion of Triacylglycerols in Black Currant and Alpine Currant Seed Oil Calculated Based on the $[M + H]^+$-Ion Abundances

Peak number	Triacylglycerol	Black currant		Alpine currant	
		Abundance	Proportion (%)	Abundance	Proportion (%)
1	52:2	63	<0.1	39	<0.1
2	52:3	2674	0.8	1127	0.9
	54:2	—	—	43	<0.1
3	52:4	1068	0.3	451	0.3
4	52:4	18927	5.5	3500	2.6
	54:3	949	0.3	263	0.2
5	52:5	14708	4.3	2394	1.8
	54:4	—	—	197	0.1
6	52:5	8293	2.4	2781	2.1
	52:6	1675	0.5	439	0.3
	54:4	6912	2.0	3589	2.7
	56:3	214	0.1	—	—
7	52:6	4843	1.4	896	0.7
	54:5	2119	0.6	647	0.5
	56:4	—	—	30	<0.1
8	54:5	26952	7.9	14038	10.6
	56:4	958	0.3	279	0.2
9	54:6	17269	5.0	6108	4.6
	56:5	316	0.1	—	—
10	54:6	56147	16.4	22916	17.3
	56:5	3003	0.9	461	0.3
11	54:7	32211	9.4	9463	7.1
	56:6	2362	0.7	242	0.2
12	54:7	50208	14.6	23500	17.7
	54:8	8200	2.4	3558	2.7
	56:6	2119	0.6	606	0.5
13	54:8	35572	10.4	11300	8.5
	54:9	2592	0.8	674	0.5
	56:7	1471	0.4	358	0.3
14	54:8	20328	5.9	11554	8.7
	54:9	6905	2.0	2540	1.9
	56:7	1009	0.3	—	—
15	54:9	6516	1.9	4740	3.6
	54:10	664	0.2	398	0.3
	56:8	337	0.1	79	0.1
16	54:9	2643	0.8	1548	1.2
	54:10	1599	0.5	972	0.7
17	54:10	993	0.3	686	0.5

lowed the comparison of the relative amounts of the corresponding triacylglycerols in the two oils studied. It should be noted, however, that the abundance of both $[M + H]^+$ and $[M - RCOO]^+$ ions has been reported to depend on the regioisomeric position of the fatty acid residues as well as on the acyl chain length and the number of double bonds of the fatty acid residues (5). Byrdwell *et al.* (8) summed the abundances of $[M + H]^+$ and $[M - RCOO]^+$ ions for quantitation purposes. This kind of approach was not considered valuable in the present study, since in several cases a single $[M - RCOO]^+$ ion was a fragment of more than one triacylglycerol. The proportions of the coeluting triacylglycerols in both of the oils resembled those reported by Manninen *et al.* (4) in the case of cSFC–FID. The proportions of triacylglycerols having a high degree of unsaturation were slightly overemphasized in cSFC–(APCI)MS, whereas the molecular discrimination in cSFC–FID at this relatively narrow ACN range has been reported to be insignificant (3). In addition, the combined proportions of isomeric triacylglycerols calculated according to the data in Table 3 were comparable to those reported in the case of direct probe mass spectrometry of black currant and alpine currant seed oil (3). According to the Tables 1–3, the proportion of triacylglycerols containing no γ-linolenic acid or stearidonic acid residues was approximately 61% in black currant seed oil and 67% in alpine currant seed oil. This was quite an expected result, since the relative amount of γ-linolenic acid and stearidonic acid measured as methyl esters is reported to be higher in black currant seed oil (3). The presence of triacylglycerols containing two γ-linolenic acid residues in black currant seed oil has been reported earlier (9,12,13). These kinds of triacylglycerols were also present in alpine currant seed oil. However, the proportion of molecules containing more than one γ-linolenic acid residue was relatively low in both oils studied.

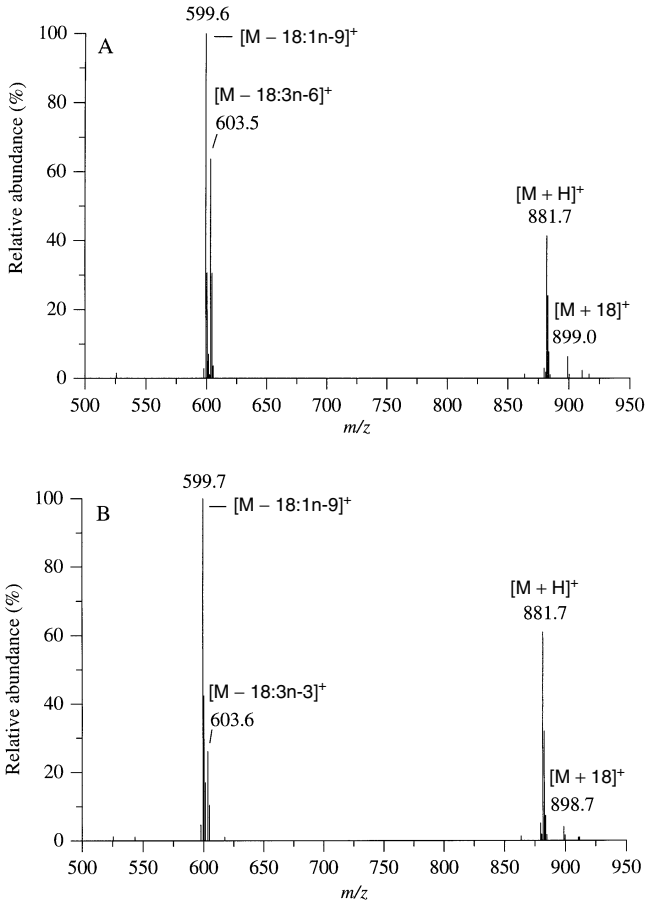

FIG. 4. The mass spectra of triacylglycerols (A) *sn*-18:1n-9-18:3n-6-18:1n-9, and (B) *sn*-18:1n-9-18:3n-3-18:1n-9.

The baseline separation of a two-component mixture of triacylglycerols *sn*-18:1n-9-18:3n-6-18:1n-9 and *sn*-18:1n-9-18:3n-3-18:1n-9 by cSFC–(APCI)MS resulted in differences in the abundances of the molecular ion $[M + H]^+$ and the $[M - RCOO]^+$ ion, produced from a loss of the linolenic acid residue, in the mass spectra (Fig. 4). The abundance of the $[M - 18:3n-3]^+$ ion was lower than that of $[M - 18:3n-6]^+$ ion, whereas the abundance of the $[M + H]^+$ ion was higher. This indicated that, together with the other factors affecting ion abundances mentioned above, the distance of the double bonds from the glycerol skeleton has an effect on the molecule stability in (APCI)MS. The proximity of the double bonds to the head of the acyl chain makes the chain a better leaving group.

The sensitivity of the cSFC–(APCI)MS method was better than that of the corresponding cSFC–FID method as the sample concentration required in cSFC–(APCI)MS was approximately four times less. The data obtained on the triacylglycerols with the used method provided valuable information on the molecular species, such as molecular weight and the molecular association of the fatty acids, which is not available with any other chromatographic detection method. The quantitative analysis of triacylglycerols with cSFC–(APCI)MS, however, will probably require separation of individual triacylglycerols, since the factors affecting the fragmentation of a molecule are very complex.

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Characterization of Polar and Nonpolar Seed Lipid Classes from Highly Saturated Fatty Acid Sunflower Mutants

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ABSTRACT: The seed lipids from five sunflower mutants, two with high palmitic acid contents, one of them in high oleic background, and three with high stearic acid contents, have been characterized. All lipid classes of these mutant seeds have increased saturated fatty acid content although triacylglycerols had the highest levels. The increase in saturated fatty acids was mainly at the expense of oleic acid while linoleic acid levels remained unchanged. No difference between mutants and standard sunflower lines used as controls was found in minor fatty acids: linolenic, arachidic, and behenic. In the high-palmitic mutants palmitoleic acid (16:1n-7) and some palmitolinoleic acid (16:2n-7, 16:2n-4) also appeared. Phosphatidylinositol, the lipid with the highest palmitic acid content in controls, also had the highest content of palmitic or stearic acids, depending on the mutant type, suggesting that saturated fatty acids are needed for its physiological function. Positional analysis showed that mutant oils have very low content of saturated fatty acids in the *sn*-2 position of triacylglycerols, between the content of olive oil and cocoa butter.

Lipids 32, 833–837 (1997).

There is currently a controversy over the health effects of semi-solid fats used in margarine and related products. Because most vegetable oils used by industries are liquid at room temperature, they must be modified by hydrogenation (1) or transesterification and then usually fractionated. Hydrogenation produces *trans* and positional isomers of fatty acids that, it has been suggested, may be related to heart disease (2,3). Transesterification increases the saturated content at position *sn*-2 of triacylglycerols, thus increasing its atherogenic effect (4). Vegetable fats, like cocoa butter, with high saturated fatty acid content but almost exclusively at positions *sn*-(1 + 3) of triacylglycerols, seem to be at least neutral on serum lipids in humans (5,6). To avoid the problems associated with chemically modified fats, a highly saturated fatty acid fat with the typical plant triacylglycerols structure should be available to the food industry.

On the other hand, a general problem found by researchers that want to study the effect of one specific fatty acid on health is the lack of a complete set of oils differing in the content of only one major fatty acid and with the same triacylglycerols structure. Oils with different fatty acid compositions from a single source could be very helpful for this purpose. In a previous mutagenesis program several sunflower mutants with either high stearic acid or palmitic acid content in normal or high oleic acid background were selected (7,8). In this work we have characterized the lipids from these mutants.

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MATERIALS AND METHODS

Plant material and growth conditions. Sunflower (*Helianthus annuus* L.) seeds from the mutant lines CAS-3, CAS-4, CAS-8, with high stearic acid content, and CAS-5, CAS-12, with high palmitic acid content (7,8) were used in this work. The control seeds were from the public lines RHA-274, with normal fatty acid content, and HA-OL9 (9) derived from Pervenets (10), with high oleic acid content. Plants were cultivated in growth chambers at 25/15°C (day/night), 16-h photoperiod, and 300 μE m^{-2} s^{-1} light intensity. Seeds for analysis were harvested 35 d after flowering and processed immediately. Three replicates were made for each experiment; a different capitulum was taken and analyzed independently for each replicate.

Lipid extraction and separation. Seeds were peeled, and then about 500 mg were ground in a screw-cap glass tube (10 × 13 mm) with a pestle and sand. Total lipids were extracted (11) and separated into triacylglycerols, diacylglycerols, and polar lipids fractions on thin-layer chromatography (TLC) silica gel plates, thickness 0.25 mm, developed with hexane/ethyl ether/ formic acid (75:25:1, by vol). The polar lipids were separated on TLC silica gel plates developed with chloroform/methanol/acetic acid/H_2O (85:15:10:3.5, by vol) or $(NH_4)_2SO_4$-impregnated silica gel plates (12) developed with acetone/benzene/H_2O (90:30:10, by vol). Individual polar lipids were identified by comparison with known standards. TLC plates were partially covered with a glass plate and exposed to iodine vapors; unexposed lipid fractions were scraped off the plates and eluted from silica with chloroform/methanol (1:2, vol/vol).

Lipase hydrolysis. For triacylglycerols fatty acid positional analysis, 10 mg of purified triacylglycerols were hydrolyzed with 2 mg pancreatic lipase (13) in 1 mL of 1 M Tris buffer pH 8, with 0.1 mL $CaCl_2$ (22%) and 0.25 mL deoxycholate (0.1%). After approximately 60% of the triacylglycerols were

hydrolyzed (1–2 min), the reaction was stopped by adding 0.5 mL of 6 N HCl. The lipids were extracted with 1.5 mL ethyl ether three times. The reaction products were separated by TLC as already indicated. The free fatty acids and *sn*-2-monoacylglycerols bands, representing, respectively, the positions 1 + 3 and 2 of triacylglycerols, were scraped off the plate and transmethylated. The procedure was checked by comparing the fatty acid composition of the original triacylglycerols and those remaining after the partial hydrolysis.

Lipid analysis. Fatty acid methyl esters were obtained from the isolated lipids (14) by heating the samples at 80°C for 1 h in a 3-mL solution of methanol/toluene/H_2SO_4 (88:10:2, by vol). After cooling, 1 mL of heptane was added and mixed. The fatty acid methyl esters were recovered from the upper phase, then separated and quantified using a Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA) with a Supelco SP-2380 capillary column (30-m length; 0.32-mm i.d.; 0.20-µm film thickness) of fused silica (Bellefonte, PA). Hydrogen was used as carrier gas. The fatty acids were identified by comparison with known standards. Unusual fatty acids (16:1 and 16:2) were identified by gas–liquid chromatography–mass spectrometry of their picolinyl esters, using electron impact ionization at 70 eV (15,16).

RESULTS

The fatty acid composition of seed lipids from the five sunflower mutants and the two control lines is shown in Table 1. The controls were RHA-274, with normal fatty acid composition, and the high oleic acid line HA-OL9, with a very low level of linoleic acid (Table 1, Part A). The palmitic acid content of the control lines was 5–7% in total and neutral lipids

TABLE 1
Fatty Acid Composition of Total Lipids (TL), Polar Lipids (PL), Triacylglycerols (TAG), and Diacylglycerols (DAG)[a]

A: Control lines		Fatty acid composition (mol%)							
Line	Lipid	16:0	16:1	18:0	18:1	18:2	18:3	20:0	22:0
RHA-274	TL	6.4	—[b]	4.9	30.8	56.2	—	0.3	1.0
	PL	12.8	—	5.3	17.7	61.9	—	0.9	1.3
	TAG	6.8	—	5.0	31.4	55.4	—	0.4	0.9
	DAG	7.1	—	3.8	25.9	62.3	—	0.5	0.4
HA-OL9	TL	5.2	—	3.7	88.1	1.5	—	0.3	1.1
	PL	11.6	0.6	5.1	77.2	3.3	0.5	0.6	1.1
	TAG	5.3	—	3.8	88.3	1.4	—	0.4	0.7
	DAG	7.4	0.4	4.3	86.2	1.5	—	—	—

B: High palmitic acid mutants										
Line	Lipid	16:0	16:1	16:2	18:0	18:1	18:2	18:3	20:0	22:0
CAS-5	TL	30.2	4.3	1.5	4.2	8.0	50.1	0.4	—	1.2
	PL	26.8	2.1	0.6	2.4	6.1	60.7	—	0.5	0.7
	TAG	30.6	4.5	1.5	4.2	7.8	49.7	0.4	—	1.1
	DAG	18.9	2.3	0.6	4.5	12.0	59.0	0.6	1.2	0.8
CAS-12	TL	27.8	7.6	—	1.7	56.7	4.4	—	0.3	1.3
	PL	17.5	3.3	0.7	3.2	63.5	11.1	0.3	—	0.4
	TAG	28.9	7.6	—	1.9	56.1	4.1	—	0.3	1.0
	DAG	18.1	5.0	—	2.9	68.2	5.3	—	—	—

C: High stearic acid mutants									
Line	Lipid	16:0	16:1	18:0	18:1	18:2	18:3	20:0	22:0
CAS-3	TL	5.4	—	26.1	14.2	51.3	—	1.4	1.3
	PL	9.6	—	18.5	11.5	57.8	0.7	1.0	0.8
	TAG	5.3	—	27.0	14.2	50.6	—	1.4	1.4
	DAG	6.4	—	16.4	16.4	59.5	—	0.6	0.4
CAS-4	TL	5.9	—	11.9	27.8	53.0	—	0.6	0.7
	PL	11.1	0.4	10.1	15.8	59.8	0.9	0.3	1.6
	TAG	6.1	—	12.5	28.1	52.2	—	0.5	0.4
	DAG	5.0	—	6.9	29.6	58.1	—	—	—
CAS-8	TL	6.3	—	11.7	18.1	61.6	—	0.6	1.3
	PL	10.0	—	12.9	10.9	63.9	—	0.4	1.9
	TAG	6.6	—	12.5	19.3	59.4	—	0.6	1.4
	DAG	6.1	—	8.7	19.5	64.9	—	—	0.6

[a]SD < 2.5% of mean value. [b]— = <0.2%.

and around 12% in polar lipids, while the content of stearic acid was 4–5% in all lipids. Linoleic acid was the main component of RHA-274, followed by oleic acid. In contrast, oleic acid represented 77–88% in the different lipids of HA-OL9. Linolenic, arachidic, and behenic acids were minor components of these lines. Two types of sunflower mutants were studied: two lines with high palmitic acid content in normal (CAS-5) and high oleic acid (CAS-12) backgrounds, respectively (Table 1, Part B), and three lines with high stearic acid content (CAS-3, CAS-4 and CAS-8) in normal background (Table 1, Part C).

The high palmitic acid mutants contained, in the total seed lipids, five times (30%) the palmitic acid normally found in control lines (Table 1, Part B). The highest increase was observed in triacylglycerols which represent approximately 94% of seed lipids (17). The palmitic acid content of polar lipids and diacylglycerols also increased, particularly the polar lipids of CAS-5 (27%). In both mutants palmitic acid increased mainly at the expense of oleic acid. The percentage of linoleic acid, however, remained almost unchanged in the different lipids of CAS-5 and increased in those of CAS-12 compared with RHA-274 and HA-OL9, respectively. Palmitoleic acid (identified as 16:1n-7), which only occurred in trace amounts in the control lines (Table 1, Part A), was present in the high palmitic mutants, mainly in triacylglycerols (4.6–7.6%). Palmitolinoleic acid (identified as 16:2n-7, 16:2n-4), which is not normally found in sunflower seed lipids, was present in these mutants in minor amounts. The other minor fatty acid components remained unchanged.

The stearic acid mutants can be divided into two types depending on the stearic acid level in total seed lipids (Table 1, Part C): one with 26% (CAS-3) and two with 12% (CAS-4 and CAS-8). The stearic acid content increased in all lipids of these mutants compared with the controls. As in the high palmitic mutants, the higher increase of stearic acid was observed in triacylglycerols (five times the value of control line RHA-274 in the case of CAS-3 and 2.5 times in the case of the other two mutants). In CAS-3 and CAS-4 the increase in stearic acid was mainly at the expense of oleic acid and, to a minor extent, of linoleic acid. In CAS-8 linolenic acid occurred at the expense of oleic acid. No changes were observed in the minor fatty acids.

Because of the very different fatty acid composition of these mutant lines it was possible to study the specificity of fatty acids for the different polar lipids. Table 2 shows the fatty acid composition of main polar lipids from control and mutant lines. The minor fatty acids have not been considered, as no differences were found either within the mutants or between these and the control lines. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, monogalactosyldiglyceride, and digalactosyldiglyceride were present as the main components of the polar lipid fraction of all lines containing high levels of linoleic acid (RHA-274, CAS-5, CAS-3, CAS-4 and CAS-8). Interestingly, phosphatidylcholine was almost absent from the lines containing high levels of oleic acid (HA-OL9 and CAS-12) and thus no reliable fatty acid analysis could be done. Monogalactosyldiglyceride and digalactosyldiglyceride had about 2–4% of linolenic acid in all cases (data not shown).

TABLE 2
Fatty Acid Composition of Polar Lipids[a]

A: Control lines		Fatty acid composition (mol%)				
Line	Lipid	16:0	16:1	18:0	18:1	18:2
RHA-274	PC	10.7	—[b]	4.3	25.3	59.2
	PI	33.8	—	14.0	14.2	37.4
	PE	13.1	—	3.1	17.3	66.2
	DGDG	7.2	—	5.4	25.1	62.3
	MGDG	11.0	—	3.6	22.4	62.9
HA-OL9	PI	20.7	0.6	6.9	65.1	6.4
	PE	4.8	0.6	1.0	88.3	4.9
	DGDG	5.6	2.0	3.8	73.9	14.7
	MGDG	4.1	0.8	1.5	87.7	5.3
B: High palmitic acid mutants						
Line	Lipid	16:0	16:1	18:0	18:1	18:2
CAS-5	PC	26.1	2.7	1.7	9.7	59.5
	PI	41.9	0.4	3.1	3.4	51.0
	PE	25.2	1.8	1.4	5.5	65.7
	DGDG	34.9	1.8	3.0	8.6	51.7
	MGDG	13.6	3.9	1.1	8.2	73.2
CAS-12	PI	36.9	0.8	3.3	54.4	4.4
	PE	22.2	2.6	0.8	69.7	4.4
	DGDG	25.5	3.2	4.0	52.4	14.9
	MGDG	16.3	4.0	1.0	70.9	7.8
C: High stearic acid mutants						
Line	Lipid	16:0	16:1	18:0	18:1	18:2
CAS-3	PC	9.5	0.4	22.4	11.5	56.2
	PI	16.0	0.1	41.4	4.9	36.9
	PE	11.5	0.2	18.9	8.1	61.3
	DGDG	5.6	—	21.9	6.1	66.4
	MGDG	9.3	0.2	17.5	13.3	59.6
CAS-4	PC	7.7	—	14.4	24.9	53.0
	PI	16.4	0.1	26.3	10.2	46.4
	PE	9.3	0.1	9.9	16.9	63.3
	DGDG	5.4	—	10.0	21.3	62.7
	MGDG	6.4	0.2	13.0	23.1	57.3
CAS-8	PC	12.5	—	16.0	15.4	55.2
	PI	17.0	—	27.5	5.4	50.1
	PE	11.6	—	10.9	13.7	63.8
	DGDG	2.3	—	7.8	19.3	69.6
	MGDG	7.9	—	20.2	13.9	57.9

[a]SD < 2.5% of mean value. Abbreviations: PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; DGDG, digalactosyldiglyceride; MGDG, monogalactosyldiglyceride.
[b]— = <0.2%.

All polar lipids from the mutants had increased palmitic (Table 2, Part B) or stearic (Table 2, Part C) acids compared with the corresponding control lines (Table 2, Part A). Nevertheless, the relative contents of these acids depended on the mutant phenotype. As a general rule an increase of palmitic or stearic acid in any lipid was accompanied by some decrease of, respectively, stearic or palmitic acid, thus increasing the total saturated fatty acid content and changing the

TABLE 3
Fatty Acid Composition of *sn*-2 and *sn*-(1 + 3) Positions of Triacylglycerols[a]

Line	Position	Fatty acid composition (mol%)				
		16:0	16:1	18:0	18:1	18:2
Control						
RHA-274	1+3	9.2	—[b]	6.1	34.0	50.7
	2	0.5	—	0.4	34.7	64.2
HA-OL9	1+3	5.1	—	5.8	87.4	1.6
	2	0.3	—	—	98.6	1.1
Mutant						
CAS-3	1 + 3	8.3	—	37.6	10.9	43.1
	2	0.4	—	1.4	22.4	75.7
CAS-4	1 + 3	9.8	—	18.8	33.9	37.3
	2	—	—	0.3	38.9	59.9
CAS-8	1 + 3	4.7	—	11.3	19.3	64.7
	2	—	—	0.3	24.1	75.4
CAS-5	1 + 3	48.3	6.6	4.4	11.8	28.7
	2	1.3	1.9	—	14.0	82.5
CAS-12	1 + 3	39.4	7.3	4.8	46.6	1.6
	2	1.4	1.6	0.8	92.3	3.2

[a]SD < 2.5% of mean value. [b]— = <0.2%.

palmitic/stearic ratio. A special behavior was observed in phosphatidylinositol. In all mutant lines it had the highest saturated fatty acid content, more than in the control lines, in which it was already high. In the case of the high palmitic mutants 16:0 increased to up to 40%, while stearic acid decreased to 3%. In the high stearic mutants there was a very high content of stearic acid, 41% in CAS-3 or 26–27% in CAS-4 and CAS-8, accompanied by a decrease in the palmitic acid content to 16%. Interestingly, the total saturated acid content in phosphatidylinositol remained fairly constant (approximately 45%) in the different lines, with the exception of the high oleic line HA-OL9 (27.6%) and the very high stearic line CAS-3 (57.4%). In the former, at least 7.4% saturated fatty acids must be at *sn*-2 position of phosphatidylinositol.

The nutritional quality of an oil is determined by its fatty acid composition as well as by the distribution of these fatty acids in triacylglycerols. Table 3 shows the positional analysis of triacylglycerols from control and mutant sunflower seeds. All sunflower lines had very low saturated fatty acid contents in the *sn*-2 position of triacylglycerols. Even in the case of the mutants with high palmitic or stearic acid content, these acids were located almost exclusively at the *sn*-(1 + 3) position. The only important fatty acids found in the *sn*-2 position were oleic and linoleic acids. As expected, in control and mutant lines with normal background, oleic acid was almost evenly distributed while linoleic acid was the main fatty acid at the *sn*-2 position. In the high oleic lines, this position was occupied almost exclusively by oleic acid (over 92%). The behavior of 16:1 in the high palmitic mutants, CAS-5 and CAS-12, was different from that of 18:1 despite the fact that both acids are Δ9 monounsaturated. This acid was mainly located at *sn*-(1 + 3) position, although it was also present in the *sn*-2 position.

DISCUSSION

The mutant sunflower lines presented here contained high levels of saturated fatty acids in the seed oil (up to 30–35%) without undesirable rearrangements in the biosynthetic pathway (Table 1). This is important, as the mutants can be used to develop commercial sunflower lines with high agronomic value. Sometimes, in using mutagenic or molecular biological techniques to modify seed fatty acid composition, some unpredictable responses have been found. For instance, mutant soybean lines with increased contents of palmitic or stearic acids (18) also produced increased levels of linolenic acid, which reduce oil stability. Transgenic canola (19) obtained using anti-mRNA construct to block stearate desaturation, thus raising the stearic acid content in the seeds, also showed increased levels of the products of stearate elongation—arachidic, behenic, and lignoceric acids (10%)—and induced linolenic acid biosynthesis (rising to 23%).

The saturated fatty acids increased in all lipid classes, mainly at the expense of oleic acid, while maintaining or even increasing linoleic acid content. The fatty acid composition of polar lipids seems to be fully compatible with the functioning of biological membranes during seed formation. The fatty acid composition seems to be critical in phosphatidylinositol. This lipid always contained high levels of saturated acids (approximately 45%, except in one case). In the normal and high oleic lines palmitic acid was the main saturated fatty acid. In the high palmitic and high stearic lines the increase of the corresponding saturated fatty acid occurred at the expense of the other. These results suggest that molecular species containing one saturated and one unsaturated acid are needed to fulfill the specific functions of phosphatidylinositol and also that the requirements should not be strict, as palmitic can be a substitute for stearic acid in CAS-3 and linoleic for oleic acid in CAS-12. Phosphatidylcholine, the main polar lipid in sunflower seed and substrate of the oleate desaturase, was practically absent from high oleic lines. By taking into account that oleoyl-phosphatidylcholine desaturase activity could not be detected in developing high oleic sunflower seeds (20), the results suggest that the availability of phosphatidylcholine was modified in the high oleic mutant. The 16:1 formation in high palmitic mutants must be due to the stearate desaturase activity inside the plastid over palmitoyl-acyl carrier protein as a substrate (21). This acid was shown to occur in all lipids of the high palmitic CAS-5 and CAS-12 lines, mainly at *sn*-(1 + 3) position of triacylglycerols. Outside the plastid, 16:1 could be desaturated to 16:2. This acid occurred in CAS-5 but not in CAS-12, thus suggesting the action of the oleoyl-phosphatidylcholine desaturase. These mutants will allow the study of polar lipid functions in seed (phosphatidylcholine in high oleic mutants or phosphatidylinositol in highly saturated fatty acid mutants) and their specificity for fatty acid composition. The different behavior of palmitoleic and oleic acids in triacylglycerols positional analysis (Table 3) shows that the specificity of the sunflower *sn*-2-glycerol-acyltransferase depends not only on the unsaturation degree but also on the chain length.

The most important quality of the oils of these mutants is that the saturated fatty acids are excluded from the *sn*-2 position of triacylglycerols (Table 3), like most vegetable oils. Fatty acids at this position are preferentially absorbed through the intestinal wall (22) while those liberated by lipase hydrolysis from positions *sn*-(1 + 3), particularly saturated fatty acids, may be excreted. Several mechanisms have been proposed to explain the preferred excretion of saturated free fatty acids, including the formation of insoluble calcium/magnesium salts or acid–calcium soap complexes (23). This is important because there is controversy about the biologic effects of the different fats. Cocoa butter contains high levels of both stearic (35%) and palmitic (25%) acids but these, as would be expected, are almost exclusively located at positions *sn*-(1 + 3). In contrast, an animal fat, namely lard, also contains high levels of palmitic (27%) and stearic (16%) acids, but at position *sn*-2 it contains 65% and 4%, respectively. Palm oil, on the other hand, contains high levels of palmitic acid (46%) and around 23% palmitic acid at position *sn*-2 (6). The impaired absorption of saturated fatty acids could explain the neutral effect of cocoa butter on serum lipids in humans (5). Interestingly, the rearrangement by interesterification of palm oil and lard increased and decreased, respectively, the negative biologic effects of these products on rats (6). The studies on the effects of the intake of saturated fatty acids have been carried out using native or interesterified fats as already indicated. Until now, no natural fat with high stearic acid content almost exclusively in positions *sn*-(1 + 3) has been available. By using oils from mutants CAS-5 and CAS-3, the effects of palmitic acid vs. stearic acid could be studied in a high linoleic background. The effects of palmitic acid in high linoleic or high oleic lines could be determined using oils from mutants CAS-5 and CAS-12. A high stearic acid line with high oleic background is being developed and could be added to these studies.

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Lipids and Ultrastructure of *Thraustochytrium* sp. ATCC 26185

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ABSTRACT: As a representative of a genus with species considered to be potential commercial producers of the nutritionally important polyunsaturated fatty acid docosahexaenoic acid (DHA), *Thraustochytrium* sp. ATCC 26185 was investigated to determine its potential for DHA production and lipid composition. Cells from liquid shake cultures contained 32% (w/w) lipid, 18% of which was nonsaponifiable lipid. The major saturated fatty acids (14:0 and 16:0) comprised up to 59% of the total fatty acids, and DHA was up to 25% after 6 d incubation. Squalene represented 63% of the nonsaponifiable lipid, and cholesterol composed 41% of the total sterols. The phospholipids expected for eucaryotic microbes were detected with phosphatidylcholine as the major phospholipid at 76% of the total. The ultrastructure of this species was similar to other *Thraustochytrium* species except that the cells did not have surface scales and they contained unusual membrane-like structures that appeared to be associated with oil formation.
Lipids 32, 839–845 (1997).

The genus *Thraustochytrium* contains unicellular, zoospore-producing species of historic uncertain taxonomic affinity that have been isolated mainly from coastal marine environments (1,2). However, the thraustochytrids recently have been placed in the stramenopila phylum Labyrinthulomycota (3).

Thraustochytrium species have attracted recent interest because they produce the polyunsaturated fatty acid docosahexaenoic acid [22:6 (4,7,10,13,16,19); DHA] (4,5), which is one of the n-3 fatty acids, along with eicosapentaenoic acid [20:5 (5,8,11,14,17)], believed to provide the health benefits associated with the consumption of certain marine fish and oils therefrom (6). DHA accumulates in the membranes of nervous, visual, and reproductive tissues (7), and is believed to be particularly important in infant nutrition (8). Since the DHA content of fish oil is variable and may not be a satisfactory long-term source of this fatty acid, and fish oil contains arachidonic acid [20:4 (5,8,11,14)] as well as eicosapentaenoic acid, alternate economic sources of this fatty acid have been sought. *Thraustochytrium* may represent such a source of DHA (4,5,9–11).

In spite of the attention given to this genus with respect to DHA production, to our knowledge other major components (e.g., sterols, phospholipids) of the lipid from these organisms have not been reported previously. Therefore, in this communication we are reporting the lipid composition of *Thraustochytrium* sp. ATCC 26185. In addition, we have observed in cells of this species the presence of unusual membrane-like structures that appear to be associated with lipid formation.

MATERIALS AND METHODS

Source of the fungus and culture conditions. Unless noted otherwise, *Thraustochytrium* sp. ATCC 26185 was cultured in 250-mL Erlenmeyer flasks containing 100 mL of the following medium at 28°C, in low intensity fluorescent light alternated diurnally with darkness, and rotary shaking at 120 rpm: 2% (wt/vol) glucose, 0.3% (wt/vol), yeast extract, and 1% (vol/vol) mineral salts solution (12) in reconstituted sea water. Inoculations were made with 5 mL of a 6-d culture grown as described above. Cells were harvested by centrifugation at $2,000 \times g$ for 25 min, washed once with 100 mL of cold reconstituted sea water, dried by lyophilization overnight, and stored at −20°C prior to extraction. Cells that separated into pellets and a floating layer when centrifuged were combined prior to drying, storage, and analysis.

Lipid extraction and estimation of total acyl lipid. Total lipid was extracted from dry cells (*ca*. 25 mg) using the procedure of Bligh and Dyer (13) that was modified according to Kates (14), but with twice the solvent volume to cell dry weight ratio as that recommended by the latter. All extraction and subsequent manipulations of the samples were conducted at low-light intensity. The amount of lipid extracted from the cells was determined gravimetrically. For monitoring changes over the course of culture development, the amount of total acyl lipid was calculated from gas chromatographic data when the methyl ester derivatives of the fatty acid methyl esters (FAME) were prepared directly from the cells after adding 23:0 as an internal standard fatty acid (see below).

Thin-layer chromatography (TLC). Individual neutral lipids were separated from polar lipids by TLC on glass plates (20 cm × 20 cm) coated with a 250-μm layer of silica gel 60

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Abbreviations: DHA, docosahexaenoic acid; FAME, fatty acid methyl esters; GLC, gas–liquid chromatography; MS, mass spectrometry; ^{31}P NMR, pulsed nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography; TMS, trimethylsilyl.

(Whatman, Maidstone, NJ). The plates were developed twice in hexane/diethyl ether/acetic acid (79:20:4, by vol), and unless the lipids were to be recovered for further analysis, the individual lipid classes were visualized in iodine vapor. For the recovery of individual lipid classes, silica gel was removed from the plates and washed with chloroform/methanol (2:1, vol/vol). Individual phospholipid classes were separated by TLC as described above except that the plates were developed in chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5, by vol). Lipid classes were identified by comparison of their R_f values with those of corresponding lipid standards (Nu-Chek-Prep, Elysian, MN).

Sample derivatization and gas–liquid chromatographic analysis (GLC). For the preparation of methyl ester derivatives of the total fatty acids, a portion of total lipid (*ca.* 5 mg) was heated with 3.0 mL of methanolic-HCl at 80°C for 1 h, and FAME were then extracted with hexane (14). In the cases where the total fatty acids were derivatized directly without extraction of the lipid, 1.5 mL methanolic base (Supelco, Inc., Bellefonte, PA) was added to about 20 mg dry cells, the mixture was heated at 70°C for 15 min, and the FAME were then recovered as before. The FAME were analyzed by gas chromatography as described previously (15), except that the gas chromatograph (Varian 3300, Palo Alto, CA) was equipped with a 30 m × 0.25 µm fused silica capillary column coated with DB-225 (50% cyanopropyl methyl 50% phenyl silicone) (J&W Scientific, Folsom, CA). Injector and detector temperatures were 250°C, and the oven temperature was programmed from 140 to 160°C at 2°C/min, 160 to 180°C at 10°C/min, and 180 to 220°C at 1°C/ min. FAME were identified by comparison of their retention times relative to methyl tricosanoate (23:0) with those of authentic standards obtained from Nu-Chek-Prep, and they were quantified by the internal standard method using 23:0 as the standard.

To obtain the nonsaponifiable lipid, a portion of the total lipid (*ca.* 200 mg) was heated for 2.5 h in 8 mL of 95% ethanol containing 0.8 mL of 33% (wt/vol) KOH (14). The nonsaponifiable lipids were removed from the hydrolysate by washing it three times with 5 mL of hexane. The combined hexane washes were washed twice with 5 mL 3% KOH, twice with 5 mL H_2O, and then brought to dryness under a stream of nitrogen gas. The amount of nonsaponifiable lipid was determined gravimetrically.

Sterols in the nonsaponifiable fraction were analyzed by gas chromatography as their trimethylsilyl ether (TMS) derivatives. Steryl-TMS derivatives were prepared by heating a portion (*ca.* 20 mg) of the nonsaponifiable lipid obtained as described above with 1 mL bis(TMS) trifluroacetamide (99:1, vol/vol) trimethylchlorosilane (Supelco Inc., Bellefonte, PA) at 70°C for 25 min. The solvent was evaporated under nitrogen gas, and the samples were diluted with hexane prior to analysis by GLC. The gas chromatograph (Hewlett-Packard model 5710, Palo Alto, CA) was equipped with a 25-m fused silica DB-5 column (Applied Science Laboratories, State College, PA). Injector and detector temperatures were 300°C and the oven temperature was 280°C isothermal. Sterols were quantitated using the TMS derivative of 5-dihydrocholesterol as the external standard.

GLC–mass spectrometry (MS). GLC–MS analysis of total sterols was performed using a VG 70E (Manchester, England) high-resolution mass spectrometer linked to a Varian 3700 gas chromatograph equipped with a 25-m DB-5 fused silica capillary column as the sample inlet, and operated in the electron impact mode at 70 eV with the source temperature at 200°C.

Pulsed nuclear magnetic resonance (^{31}P NMR) analysis of phospholipids. About 60 mg of total lipid was dissolved in 25 mL chloroform and washed with 25 mL of the K^+/Na^+ salt of EDTA in separatory funnel by shaking vigorously at 30-min intervals over 4 h. The K^+/Na^+ EDTA salt (0.2 M, pH 6.0) solution was prepared according to Seijo *et al.* (16). Emulsions formed during the washing process were disrupted by the addition of KCl (*ca.* 2 g). The lower chloroform phase containing the lipid was collected, and the solvent evaporated under nitrogen gas. The lipid obtained after washing was dissolved in 0.5 mL of chloroform (reagent grade) containing 5% benzene-d_6 and 3.1 mg of trimethylphosphate as an internal reference followed by the addition of 0.25 mL methanol (reagent grade) containing 0.2 M aqueous Cs-EDTA at pH 6.0. The solution was mixed by vortexing, filtered through a 0.2 µm Acrodisc nylon filter (Gelman Sci., Ann Arbor, MI), and then transferred to a 5-mm NMR tube (Wilmad Glass Co., Buena, NJ). ^{31}P NMR analysis was conducted using a Bruker 250 AM system (Karlsruhe, Germany) operated at 101.3 MHz. Cs-EDTA phospholipid NMR reagent was prepared according to Meneses and Glonek (17).

Electron microscopy. Cell pellets were preserved in a 1:1 (vol/vol) mixture of growth medium and fixative (glutaraldehyde in sodium cacodylate buffer) such that the final concentrations were 2% glutaraldehyde in 0.05 M buffer (pH 7.0). The duration of fixation was 4 h, the first 30 min at room temperature, and the remainder of the time at 4°C. This was followed by one wash of the cells in growth medium and 0.1 M buffer (1:1, vol/vol) and two washes in 0.05 M buffer. All buffer washes were at 4°C. Postfixation was carried out in cold OsO_4 (1%) in 0.05 M buffer for 3 h. Following washes in cold buffer and then distilled water, pellets were embedded in 2% agar and, when necessary, diced into 1-mm cubes. Cells were then stained en bloc in 0.5% aqueous uranyl acetate for 2 h at 4°C, dehydrated in an ethanol/propylene oxide series, and embedded in Spurr's resin (18). Ultrathin sections (65 nm) were made, mounted on nickel grids, and stained further with uranyl acetate and lead citrate. Specimens were viewed with a Zeiss 10 transmission electron microscope (Thornwood, NY) at 60 kV.

RESULTS AND DISCUSSION

Growth and lipid content. Biomass production of *Thraustochytrium* sp. in shake flask culture increased linearly over the first 4 d of incubation and peaked at 7.5 g/L at 7 d after inoculation. The solvent extractable lipid content of cells cul-

tured under these conditions for 6 d was 32% of the cell dry weight, 18% of which was nonsaponifiable lipid. When grown under the same conditions, but without basal salts in the medium, biomass was reduced by 52%, lipid content by 14%, and nonsaponifiable lipids by 68% compared to cultures grown with the basal salts. Bajpai *et al*. (5) found that *T. aureum* (ATCC 34304) produced up to 25% lipid while Kendrick and Ratledge (19) reported that the same strain of *T. aureum*, and *T. roseum*, produced only 10 and 0.5% total lipid, respectively.

Thraustochytrium species have been reported to be responsive to light (20). When grown in yeast extract/glucose medium containing basal salts with low-intensity diurnal, fluorescent light, the cultures were a pale yellow color. The cultures of *Thraustochytrium* sp. grown in the light (538 lux) were orange. Also, cultures from the light had almost twofold more biomass than cultures incubated for 6 d in the dark, but there was essentially no difference in the total lipid or the relative proportion of DHA (data not shown).

The total fatty acid profile of *Thraustochytrium* sp. was characterized by the following: (i) relatively high saturated fatty acid content (14:0, 15 to 19%; 16:0, 32.0 to 41%), (ii) very low proportions of 18 and 20 carbon fatty acids, and (iii) high 22 carbon chain length polyunsaturated fatty acid (PUFA) content and DHA being the predominant PUFA at 25 to 32% of the total fatty acids over the 13-d growth period. Since there was little change in the proportion of fatty acids over the 13-d growth period, the fatty acid composition of cells from 6-d-old cultures in Table 1 is considered to be representative. In such cultures, the relative amounts of DHA in the polar lipid and triacylglycerols were similar (data not given).

TABLE 1
Total Fatty Acid Composition of *Thraustochytrium* sp. ATCC 26185

Fatty acid[a]	Mole %
12:0	0.6
13:0	0.1
14:0	18.2
15:0	2.3
15:1	<0.1
16:0	40.7
16:1	0.7
17:0	0.4
17:1	<0.1
18:0	1.1
18:1n-?	0.5
18:1n-9	0.5
18:3n-3	<0.1
18:3n-6	0.1
20:0	0.1
20:3n-6	0.2
20:4n-6	0.2
20:4n-3	0.4
20:5n-3	0.5
22:0	<0.1
22:4n-6	<0.1
22:5n-6	7.6
22:5n-3	0.3
22:6n-3	25.0
Others	0.4

[a]18:2, 20:1, 20:2, 22:1, 22:2, and 22:3 were not detected.

Shaw (21) recognized that the more primitive fungi, including those taxa now classified as stramenopiles and no longer considered to be fungi, possess both the n-3 and n-6 pathways of PUFA biosynthesis which are both operational in *Thraustochytrium* sp. DHA is the apparent end product of the n-3 pathway, and 22:5 (4,7,10,13,16) appears to be the end product of the n-6 pathway. Previously, a 22:5 with unspecified double-bond location (4) and 22:5 n-3 (19) have been reported for *T. aureum*. The two isomers of 22:5 can be separated by GLC, and the retention time of an authentic 22:5n-6 standard matched that from *Thraustochytrium* sp. Also, its identity as a 22:5 pentaene has been confirmed by GLC–MS (data not shown). Eucaryotic microbes generally are not known to produce n-6 PUFA beyond 20 carbon atoms and four double bonds (22,23).

Squalene and sterols. The identity of squalene as the major component of the nonsaponifiable fraction (Table 2) was confirmed by comparison of GLC retention times of the sample and an authentic standard, and by GLC–MS. The mass spectrum of this compound was similar to that of a squalene standard with a molecular ion at *m/z* 410. Squalene is not known to typically accumulate in such high amounts in fungal cells, i.e., 35 mg squalene per g dry weight of cells; however, it was a major component of lipid from *Rhizopus arrhizus* grown in liquid shake culture (24).

Sterols represented 38% of the nonsaponifiable lipid. At least 13 substances detected in *Thraustochytrium* sp. by GLC are believed to be sterols. The major sterol was cholesterol at 41% of the total sterols (Table 2). The mass spectrum of this compound matched that of a standard TMS derivative of cholesterol and was consistent with previously published spectra of this sterol (25).

The component representing 7.6% of the total sterols had a molecular ion at *m/z* 470, suggesting that it was a C_{28} diene, and a fragmentation pattern shown in Table 2. Based on the prominent $M^+ - 129$ fragment, the nuclear double bond is believed to be at the C-5(6) position, and the second double bond in the side chain, probably the C_{22} rather than C-24(28) position since fragments at *m/z* 296 or 386 were not detected (see below). An additional C_{28} diene was detected at 1.5% of the total sterols with double bonds in the C-5(6) and C-24(28) positions. The latter double-bond assignment is based on the presence of strong fragment ions at *m/z* 296 and 386 (25).

The molecular ion at *m/z* 484 for the sterol representing 21% of the total sterols suggested that this sterol is a C_{29} diene. The GLC retention time and mass spectrum were similar to that of the TMS derivative of an authentic 24-ethylcholesta-5,22-dienol standard, and the mass spectrum was similar to a previously published mass spectrum of the TMS derivative of this sterol (25).

The component representing 12% of the total had a molecular ion of *m/z* 482, suggesting that it is a C_{29} triene. The mass spectrum of this sterol had several major fragment ions anal-

TABLE 2
Squalene and Sterols of *Thraustochytrium* sp. ATCC 26185

Component	Major fragment ions (*m/z*)	Relative percentage[a] (%)
Squalene	410 (M^+)	62.6 —
Sterols		
Cholest-5-enol (cholesterol)	458 (M^+), 368, 353, 329, 255, 129 (100%)	15.1 (41.3)
24-Methyl-cholesta-5,22-dienol (7-dihydroergosterol)	470 (M^+), 380, 365 351, 341, 325, 255 129 69 (100%)	2.8 (7.6)
24-Methyl-cholesta-5,24 (28)-dienol (24-methylene Cholesterol)	470 (M^+) 386, 380, 365, 343, 341, 296, 281, 129 (100%)	0.5 (1.5)
24-Ethyl-cholesta-5,22-dienol	484 (M^+), 394, 379, 355 ($M^+ - 129$), 351 255, 213, 129, 83	4.5 (12.4)
24-Ethyl-cholesta-5,7,22-trienol	482 (M^+), 392, 394, 377 (100%) 351, 253	8.5 (20.7)
Unknowns (8)		6.0 (16.5)

[a]Values are the percentage of total nonsaponifiable fraction; those in parentheses are percentage of total sterols.

ogous to those of the TMS derivative of ergosterol ($C_{28}\Delta^{5,7,22}$) (25) except with 14 higher mass units. For example, *m/z* 377 is analogous to *m/z* 363 in the mass spectrum of ergosterol, *m/z* 351 (100%) analogous to *m/z* 337, and *m/z* 392 to *m/z* 378. Therefore, in the absence of *m/z* 296 and 386, which are indicative of a C-24(28) double bond, this sterol may be the C_{29} analogue of ergosterol, i.e., 24-ethyl-cholesta-5,7,22-trienol. This sterol, described as 7-dehydrostigmasterol, has been detected in the soil amoeba *Acanthamoeba* (26).

The sterol composition of several other *Thraustochytrium* species *(T. roseum, T. striatium, T. virsugence,* and *T. aureum)* was very similar to that of *Thraustochytrium* sp. (data not given).

Phylogenetically primitive, eucaryotic microorganisms vary considerably in the dominant sterol both within and between the various major taxa (15,27). Ergosterol, which is considered to be the "fungal sterol" (28), is characteristically absent from the stramenopila such as Oomycota and Hypochytridiomycota where dominant sterols are cholesterol and/or Δ^5 C_{28} and/or Δ^5 C_{29} sterols with or without double bonds in other positions (28–30). Otherwise, there seems to be no apparent pattern to sterol distributions among these taxa except that fucosterol [24-ethyl-cholesta-5, 24(28E)-dienol] seems to be found in the Oomycota as well as phaeophycean algae (27).

Phospholipids. Phospholipid represented about 8% of the total lipid of *Thraustochytrium* sp. cells from 6-d cultures. The major phospholipid was phosphatidylcholine (PC) at 76% of the total phospholipids (Table 3). Other phospholipids detected were phosphatidylethanolamine (PE) (2%), phosphatidylglycerol (1%), and diphosphatidylglycerol (4%). Lysophosphatidylcholine and phosphatidylinositol were not resolved by ^{31}P NMR in this study, but collectively represented 17% of the total. The presence of both of these phospholipids was confirmed by TLC (data not shown). Phosphatidic acid was not detected in cells cultured in yeast extract/glucose medium with mineral salts, but it was a minor component of cells grown in other media. PC and PE are the most common eucaryotic microbial phospholipids but vary widely in relative proportions between species (23). It is unusual, but not unknown, for PC to be in such high relative amounts and PE to be in such low amounts.

Ultrastructure. Thraustochytrium sp. cells were heterogeneous in size and appearance, with diameters ranging from about 6 to 21 µm, and the cytoplasm appeared granular or filled with multiple oil globules. Cells collected at 6 d after inoculation were multinucleate, spherical unicells (Fig. 1A and B). Up to nine nuclear profiles were observed in a given thin section of a single cell. The presence of condensed chromosomes and spindle fibers in some sections indicated that some of the cells were undergoing mitosis when fixed, and light microscope observations indicated that such coenocytes eventually released smaller cells (data not shown).

Many, but not all, subcellular features of this species were similar to those observed previously for other species of this genus with numerous mitochondrial profiles containing tubular cristae (1,32) (Fig. 1 A and C). Each nucleus was associ-

TABLE 3
Phospholipids of *Thraustochytrium* sp. ATCC 12685

Phospholipid	Chemical shift[a] (ppm)	Relative percentage (%)[b]
Phosphatidylcholine	–0.84	76.2 ± 0.1
Lysophosphatidylcholine + phosphatidylinositol	–0.35	16.9 ± 2.5
Phosphatidylethanolamine	0.08	2.3 ± 0.6
Phosphatidylglycerol	0.54	1.3 ± 0.8
Diphosphatidylglycerol	0.22	3.7 ± 0.7

[a]Chemical shift values are calculated relative to that for phosphatidylcholine assigned the average value of –0.84. Amounts of individual phospholipids were determined using the internal standard Triphenylphosphate.
[b]Values are the averages of two analyses plus/minus the standard deviation.

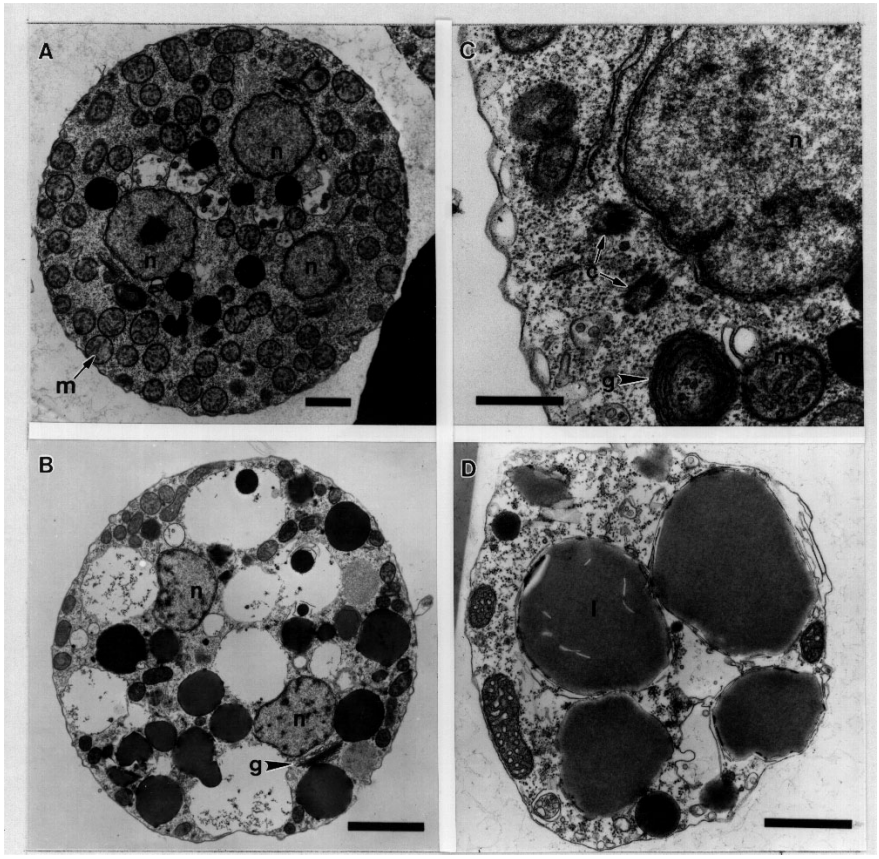

FIG. 1. Transmission electron micrographs of *Thraustochytrium* sp. ATCC 26185 cells. The cells are multinucleate (n, panels A and B) and also contain mitochondria (m) with tubular cristae, Golgi bodies (g), and centrioles © (panels B and C). Prominent lipid bodies (1, panel D) exist in all cells. Bar = 1 μm in panels A and D, 2 μm in panel B, 0.5 μm in panel C.

ated with centrioles (Fig. 1C) and Golgi bodies (Fig. 1 B and C). Species of *Thraustochytrium* are known to form rhizoids or ectoplasmic nets from organelle complexes known as sagenogenetosomes (1,32), but the cells of this species exhibited no rhizoids either in liquid medium or when cultured on semisolid agar medium. Also, the cells were naked with neither cell walls nor scales covering the plasma membrane. This latter observation is of interest in that Goldstein *et al*. (31) observed that the plasma membrane of *T. aureum* was covered by a wall consisting of "tangentially arranged laminations." Darley *et al*. (33) showed that the wall of an unnamed *Thraustochytrium* species consisted of circular scales manufactured within the Golgi apparatus, and that the composition of the scales differed from those of the closely related *Schizochytrium aggregatum*. It is possible , although unlikely, that scales were present in *Thraustochytrium* sp. but were detached during centrifugation to harvest the cells. Experiments are underway to examine this possibility.

Consistent with the results of lipid analyses described above, the cells contained numerous cytoplasmic bodies (Fig. 1D) which have been confirmed as lipid bodies by staining with Sudan Black B. These lipid bodies were associated with branched, hollow membrane-like structures that appeared crystalline in their angularity (Fig. 2A). These structures seemed to be associated with lipid synthesis and deposition (Fig. 2A) and, with continued lipid deposition, they became internalized within the lipid bodies (Fig. 2B). Detailed sectional views (Fig. 2C) indicated that the boundary layer of these structures was not a true unit membrane. Instead, it appeared as electron-opaque lines less than one-half the thickness of the plasma membrane. As the structures became internalized during lipid body enlargement, their boundaries became indistinct (Fig. 2D) which seemed to be followed by loss of the internalized structures (Fig. 1D).

The presence of lipid bodies (or osmiophilic droplets) in *Thraustochytrium* and related genera has been observed by light (20,34) and transmission electron (1,31) microscopy. However, until now, no mention has been made of the membrane-like structures in species of this genus. Whether they are unique to the organism of this study is not known, but they are found consistently in cells cultured in different media and fixed at different times during the growth cycle.

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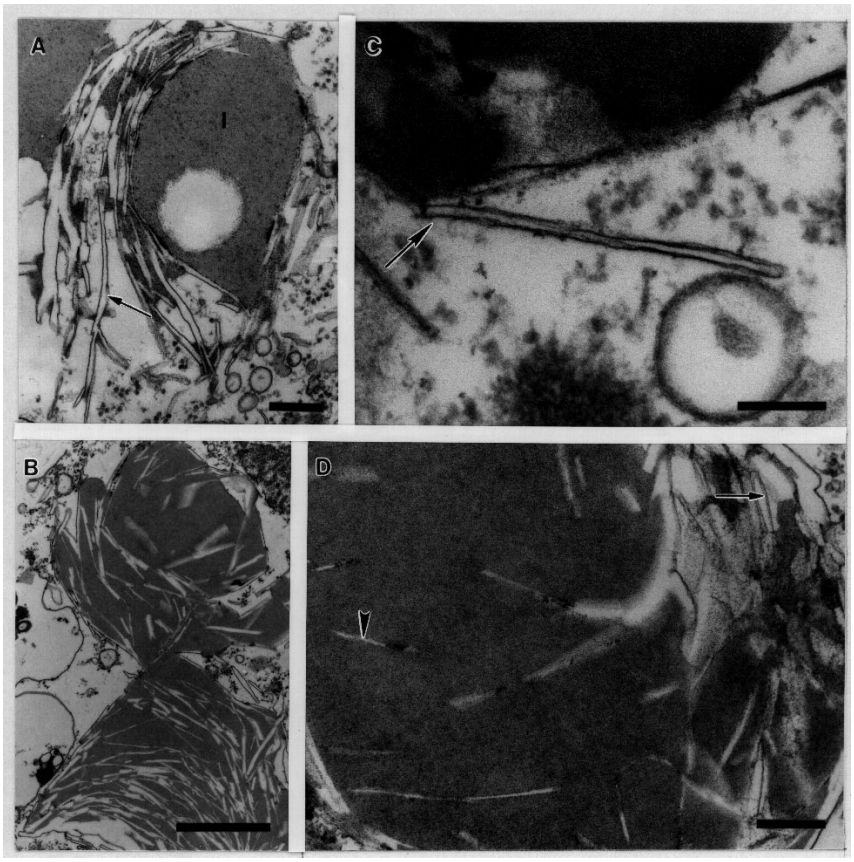

FIG. 2. Micrographs of lipid bodies (l) from cells of *Thraustochytrium* sp. 26185. Branching, hollow structures are associated with lipid synthesis (arrow, panel A) and become internalized as lipid deposition continues (panel B). The boundaries of these structures are not unit membranes (arrow, panel C) and disappear with internalization (arrow, panel D). Bar = 0.2 μm in panels A and D, 1 μm in panel B, 0.1 μm in panel C.

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On the Interrelationship Between Hepatic Carnitine, Fatty Acid Oxidation, and Triglyceride Biosynthesis in Nephrosis

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ABSTRACT: The nephrotic syndrome is associated with disturbances in plasma lipid pattern and metabolism. However, the reason for these perturbations is poorly understood. In the present study, we have investigated hepatic triglyceride metabolism in puromycin aminonucleoside-induced nephrotic syndrome in rats. Nephrotic rats displayed a 70% increase in hepatic triglyceride levels compared to controls (16.9 ± 1.6 vs. 9.8 ± 0.6 μmol/g liver; means ± SEM, $P < 0.01$). The capacity for hepatic mitochondrial β-oxidation of fatty acids was substantially elevated (80%). This was associated with a rise in the liver content of the fatty acid carrier carnitine (1.24 ± 0.06 vs. 0.85 ± 0.07 μmol/g dry weight, $P < 0.05$). A positive correlation between the levels of acetylcarnitine and acetyl-CoA was found in normal as well as in nephrotic rats, implying that carnitine plays an important role as an acetyl group acceptor in the liver under normo- and hyperlipidemic conditions. Changes in carnitine levels seem to be tightly coupled to the rate of fatty acid oxidation. There was a significant elevation in the activity of phosphatidate phosphohydrolase (E.C. 3.1.3.4) in liver microsomes from nephrotic rats (1.07 ± 0.09 vs. 0.81 ± 0.04 nmol/min·mg protein, $P < 0.02$). Hepatic very low density lipoprotein (VLDL)–triglyceride secretion rate was 18% higher in nephrotic rats than in controls. The results demonstrate a deranged hepatic triglyceride metabolism in nephrosis, with an increased hepatic triglyceride biosynthesis, a sizable accumulation of hepatic triglycerides, and only a modest increase in VLDL triglyceride secretion. In addition, mitochondrial β-oxidation of fatty acids was enhanced, associated with an increased availability of carnitine.
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Nephrotic syndrome is a condition associated with profound disturbances in lipid and lipoprotein metabolism (1–4). In addition to its clinical implications related to premature atherosclerosis, coronary heart disease and progressive renal failure (5–7), this condition provides a useful tool to investigate the regulation of hepatic lipid metabolism in a deranged state.

It has previously been suggested that hepatic triglyceride biosynthesis is enhanced in nephrosis, based on the observation that the incorporation of fatty acids into neutral lipids is increased in liver slices from nephrotic rats (8). This is of particular interest because growing evidence points to the overall importance of triglyceride availability for hepatic lipoprotein secretion (9,10). It is generally believed that under normal conditions the rate of triglyceride biosynthesis is governed by the activities of the enzymes involved in this pathway, especially phosphatidate phosphohydrolase and by the availability of fatty acid substrates (11). The latter is in turn affected by the rate of fatty acid oxidation. We have shown that β-oxidation stimulating agents reduced hepatic and plasma triglyceride levels and hepatic triglyceride output in nephrotic and normal rats (12,13), indicating that modulation of fatty acid oxidation influences triglyceride metabolism also in the nephrotic state. However, the impact of nephrosis on hepatic fatty acid oxidation has received scant attention.

The oxidation of long-chain fatty acids is known to be dependent on carnitine for their transport across the inner mitochondrial membrane, which is impermeable to CoA derivatives. It has been shown that adequate carnitine concentrations in the liver are necessary for intact mitochondrial fatty acid oxidation and ketogenesis (14). Furthermore, treatment with carnitine ameliorated alcohol-induced hepatic steatosis (15,16), suggesting that carnitine availability could influence hepatic triglyceride metabolism, and at least under some conditions be a limiting factor. However, the possible impact of nephrosis or other hyperlipidemic conditions on carnitine metabolism has not been previously addressed.

In the present work, we have studied regulatory aspects of hepatic triglyceride biosynthesis and fatty acid catabolism in experimental nephrotic syndrome. We present evidence that the liver capacity for fatty acid oxidation is elevated in nephrotic syndrome and that increased carnitine availability may contribute to this effect. Furthermore, triglyceride bio-

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Abbreviations: CPT, carnitine palmitoyltransferase; PAN, puromycin aminonucleoside; PAP, phosphatidate phosphohydrolase; VLDL, very low density lipoprotein.

synthesis was enhanced in nephrosis, associated with an increased phosphatidate phosphohydrolase (PAP) activity.

EXPERIMENTAL PROCEDURES

Male rats of the Sprague-Dawley strain weighing 200–250 g were housed in metal cages and maintained on a 12-h light–dark cycle with the light period between 6 a.m. and 6 p.m. The animals were allowed to move freely in the cages and had free access to standard chow and water. Rats used for the determination of very low density lipoprotein (VLDL)–triglyceride secretion were fasted overnight to avoid interference from chylomicrons. All other experiments were carried out in the nonfasting state. The study was approved by the ethics committee for animal experiments in Stockholm.

Nephrosis was induced by a single intraperitoneal injection of puromycin aminonucleoside (PAN) (Sigma Chemical Co., St. Louis, MO) in a dose of 100 mg/kg body weight. One week after the injection, rats were transferred to individual metabolic cages, and urine was collected for 24 h. Plasma and urine albumin concentrations were measured by an immunonephelometric method using rat albumin antibodies (Nordic Immunology, Tilburg, The Netherlands). Nephrosis was confirmed by the presence of hyperlipidemia, low plasma albumin, and urine albumin loss exceeding 250 mg/24 h. In order to ensure a well-established nephrotic syndrome and to avoid possible direct effects of PAN (17), all experiments were performed at least 6 d after PAN injection. Except for the time-course study, the rats were killed 11–13 d after PAN injection. In the time-course study four subgroups of PAN-treated rats were killed 6, 9, 12, and 14 d after the injection.

On the day of sacrifice, blood sampling was performed by open heart puncture under neuroleptic anesthesia with fentanyl + fluanisone (Hypnorm®; Janssen Pharmaceutica, Beerse, Belgium) aided by the muscle relaxant diazepam (Stesolid®; Dumex Ltd., Copenhagen, Denmark). Collection of plasma and liver tissue samples was carried out between 9 and 11 a.m.

Plasma triglyceride and cholesterol levels were determined using enzymatic procedures (18; Cholesterol CHOD-PAP method BM/Hitachi 917 System Pack [kit insert], 1994 edition; Boehringer Mannheim GmbH, Mannheim, Germany. Triglycerides GPO-PAP Boehringer Mannheim Systems [kit insert], 1995 edition; Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany). Triglyceride and cholesterol concentrations also were measured in a total lipid extract of a 20% liver homogenate. Lipid extraction was done using 20 vol of chloroform/methanol (2:1, vol/vol).

A portion of liver tissue was rapidly frozen in liquid nitrogen, freeze-dried, dissected free of connective tissue and blood, powdered, and precipitated with 0.5 mmol/L perchloric acid in 1 mmol/L Na_2EDTA and neutralized with 2.1 mol/L $KHCO_3$. Total acid-soluble carnitine, free carnitine, acetylcarnitine, and acetyl-CoA were assayed in the supernatant by enzymatic radioisotopic methods (19–21). Short-chain acylcarnitine was calculated as the difference between total acid-soluble and free carnitine.

Livers from individual rats were homogenized, and subcellular fractionation yielding mitochondrial, microsomal, and cytosolic fractions was carried out as described in detail elsewhere (22,23). Cytosolic and microsomal PAP activities were determined by measuring the conversion of ^{14}C phosphatidate into diglycerides (24). Mitochondrial β-oxidation was measured in short-term incubations as the conversion of [1-^{14}C] fatty acids in palmitoyl-CoA or palmitoylcarnitine into acid-soluble radioactive products (25). When palmitoyl-CoA was used as the substrate for oxidation, carnitine was added to the assay mixture (25). Protein concentration was measured by the Lowry procedure using bovine serum albumin as standard (26).

VLDL–triglyceride secretion rate was estimated using Triton WR 1339 (Sigma Chemical Co., St. Louis, MO) as previously described (27,28). In brief, 1 mL of a 20% solution of Triton WR 1339 (Sigma Chemical Co.) in 0.9% NaCl was intravenously injected, and blood samples were drawn hourly for 3 h after the injection. Plasma triglycerides were measured as above, and the entry rate of triglycerides into the circulation (mmol/L/h) was calculated. Data were evaluated by Student's *t*-test and linear regression analysis.

RESULTS

Induction of nephrosis by PAN resulted in a marked increase in triglyceride content in the liver (72%) while cholesterol levels remained unchanged (Table 1). Consequently, the hepatic triglyceride/cholesterol ratio increased significantly (Table 1), indicating that nephrosis primarily resulted in a derangement of hepatic triglyceride metabolism. It is well known that fatty acid β-oxidation affects the availability of fatty acids for esterification and triglyceride formation. β-Oxidation was therefore measured in the hepatic mitochondrial fraction using two substrates in parallel, palmitoyl-CoA or palmitoylcarnitine. Palmitoyl-CoA is dependent for its transport into the mitochondria on the enzyme carnitine palmitoyltransferase while this is not the case for palmitoylcarnitine. As shown in Figure 1, the mitochondrial β-oxidation activity, as measured by the formation of acid-soluble products, was increased from both substrates in livers from nephrotic rats

TABLE 1
Hepatic Lipid Levels in Nephrotic Rats and Normal Controls[a]

	Control	Nephrotic
Triglycerides (μmol/g liver)	9.8 ± 0.6	16.9 ± 1.6[b]
Cholesterol (μmol/g liver)	9.5 ± 0.2	10.1 ± 0.3
Triglyceride/cholesterol ratio	1.03 ± 0.06	1.65 ± 0.15[b]

[a]Values are means ± SEM for seven nephrotic rats and five controls.
[b]$P < 0.01$. Rats were killed 12–13 d after puromycin aminonucleoside (PAN) injection.

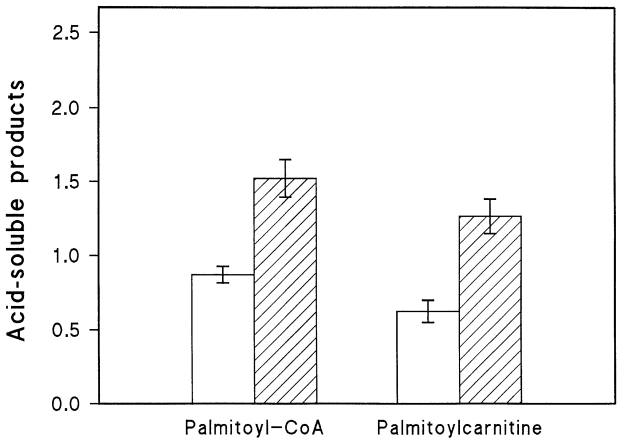

FIG. 1. Effect of nephrosis on mitochondrial β-oxidation of fatty acids. Fatty acid β-oxidation was measured as the formation of acid-soluble products (nmol/min·mg protein) from ^{14}C palmitoyl-CoA or palmitoylcarnitine. Hatched bars represent nephrotic rats (n = 7) and open bars represent normal controls (n = 4). The rats were sacrificed 12–13 d after puromycin aminonucleoside injection. Values are means ± SEM, $P <$ 0.005, with both substrates.

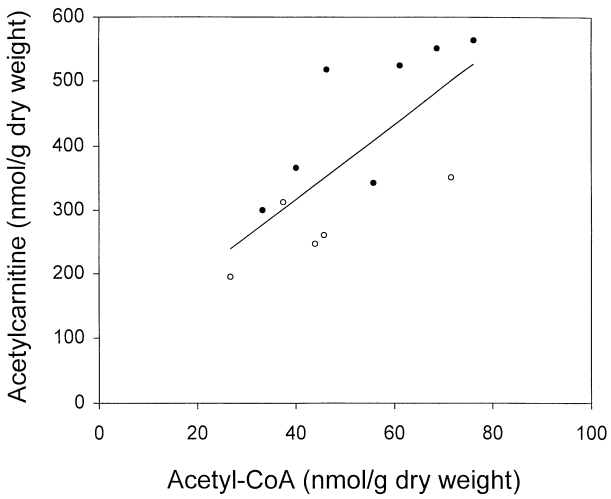

FIG. 2. Relationship between the content of acetylcarnitine and acetyl-CoA in the liver. Data are from the experiment described in Table 2. The regression line for all values (drawn) is [acetylcarnitine] = 5.8[acetyl-CoA] + 84, r = 0.72, $P < 0.01$. The regression lines were for nephrotic rats (●) [acetylcarnitine] = 5.6[acetyl-CoA] + 146, r = 0.78 and for controls (○) [acetylcarnitine] = 2.9[acetyl-CoA] + 141, r = 0.81, respectively.

compared to control rats (87 and 74%, respectively). These results indicated an increased capacity for fatty acid oxidation in the nephrotic state. To further investigate the role of the fatty acid carrier carnitine in β-oxidation of fatty acids, hepatic carnitine content was measured and, as shown in Table 2, total carnitine levels were significantly increased in nephrotic rats compared to controls. Both the free and acylated forms of carnitine were increased in nephrosis. In both experimental groups, the short-chain acylcarnitine pool consisted almost exclusively of acetylcarnitine (Table 2). Under normal conditions, acetylcarnitine is of importance as an acetyl group acceptor in muscle tissue. It was therefore of interest that hepatic acetylcarnitine and acetyl-CoA levels were positively correlated ($P < 0.01$) (Fig. 2) in both normal and nephrotic rats, indicating that the acetyl group acceptor function of carnitine is important in the liver, also in the presence of a massive derangement of hepatic triglyceride metabolism.

The aforementioned experiments were performed in the presence of established hyperlipidemia, 12–13 d after the induction of nephrosis. However, it has been shown that fatty acid synthesis changes over time after the induction of nephrosis (17). It was therefore of interest to study whether this was also the case for fatty acid oxidation. For this purpose, groups of nephrotic rats were sacrificed at different times following the induction of nephrosis. As shown in Figure 3, a slight increase in fatty acid oxidation was already observed 6 d after induction. The capacity for fatty acid oxidation increased over time, and a peak was reached at day 12.

TABLE 2
Hepatic Carnitine and Acetyl-CoA Levels in Nephrotic Rats and Normal Controls[a]

	Control	Nephrotic
Carnitine (μmol/g dry weight)		
Total acid-soluble	0.85 ± 0.07	1.24 ± 0.06[b]
free	0.52 ± 0.08	0.77 ± 0.05[b]
short-chain acyl	0.33 ± 0.03	0.47 ± 0.03[b]
acetyl	0.27 ± 0.03	0.45 ± 0.04[b]
Acetyl-CoA (μmol/g dry weight)	0.045 ± 0.007	0.054 ± 0.006

[a]Values are means ± SEM for seven nephrotic rats and five controls.
[b]$P < 0.05$. Rats were killed 12–13 d after PAN injection. See Table 1 for abbreviation.

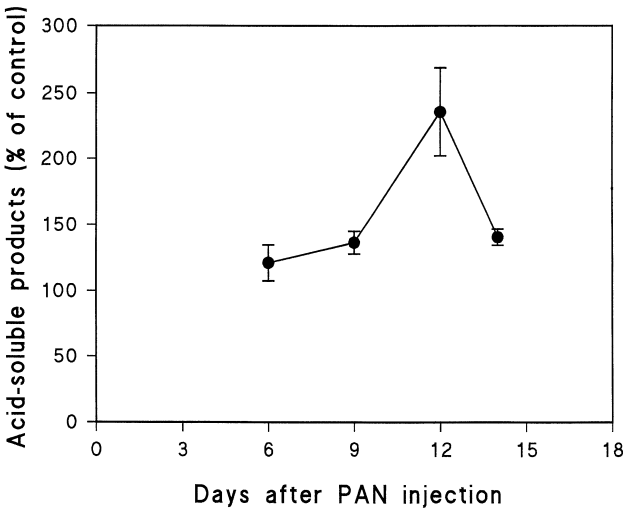

FIG. 3. Effect of nephrosis on mitochondrial β-oxidation of fatty acids: a time-course study. Fatty acid β-oxidation was measured as the formation of acid-soluble products from ^{14}C palmitoylcarnitine. Three nephrotic rats and three controls were used each day except for day 6 when there were only two rats in the nephrotic group, and day 12 with two rats in the control group. The values given for each day represent mean and range. PAN, puromycin aminonucleoside.

TABLE 3
A Time-Course Study of the Effect of Nephrosis on Hepatic Carnitine Levels[a]

	Total acid-soluble carnitine (μmol/g dry weight)			
Days after PAN injection	6	9	12	14
Control rats	0.98	0.85	0.78	1.01
	0.81	0.71	0.74	0.82
	0.70	1.01		0.95
Mean	0.83	0.86	0.76	0.93
Nephrotic rats	1.29	1.91	2.11	1.05
	1.50	2.39	1.08	0.98
		1.38	1.01	1.12
Mean	1.40	1.89	1.40	1.05

[a]Data are from the same experiment as in Figure 3. Individual values for the nephrotic rats and normal controls are given ($n = 11$ in each group). Pooled values from day 6 to day 12 were 1.60 ± 0.17 for nephrotic rats and 0.83 ± 0.05 for controls, means ± SEM, $P < 0.001$. See Table 1 for abbreviation.

TABLE 4
Effect of Nephrosis on Phosphatidate Phosphohydrolase (PAP) Activity[a]

PAP activity (nmol/min·mg protein)	Control	Nephrotic
Cytosolic	0.18 ± 0.01	0.21 ± 0.02
Microsomal	0.81 ± 0.04	1.07 ± 0.09[b]

[a]Combined results from four experiments are shown. Values are means ± SEM for 23 nephrotic rats and 21 controls.
[b]$P < 0.02$. Rats were killed 11–13 d after PAN injection. See Table 1 for other abbreviation.

Consequently, the difference between nephrotic and control rats decreased rapidly. Interestingly, the same temporal pattern was observed using either palmitoylcarnitine (Fig. 3) or palmitoyl-CoA (not shown) as substrates. The increase in palmitoylcarnitine and palmitoyl CoA levels on day 12, about twofold, was in excellent agreement with the results in Figure 1.

Hepatic carnitine levels also showed pronounced changes over time in nephrotic rats with the highest levels reached around day nine (Table 3). Except for day 14, hepatic carnitine was consistently higher in nephrotic rats compared to controls. A significant difference in carnitine levels between the two groups was observed when values from rats killed on days 6 to 12 were pooled (1.60 ± 0.17 vs. 0.83 ± 0.05, $P < 0.001$). Changes in either free carnitine or acetylcarnitine levels followed the same pattern as total carnitine (not shown).

Since triglycerides are known to play an important role in VLDL synthesis and secretion (9,10,29,30), we examined whether hepatic VLDL–triglyceride secretion was altered in nephrotic rats. For this purpose, a group of rats was fasted overnight to avoid interference from chylomicrons and intravenously injected with Triton WR 1339, which blocks the action of lipoprotein lipase on triglyceride-rich lipoproteins (27). The accumulation of plasma triglycerides under these conditions, reflecting the entry rate of newly synthesized VLDL triglycerides, was found to be 18% higher in nephrotic rats than in controls (5.3 ± 0.4 vs. 4.5 ± 0.2 mmol/L/h, $n = 6$, $P < 0.05$), well in line with earlier observations (31). This modest increase was in contrast with the severe elevations of plasma triglyceride levels (19.8 ± 3.2 vs. 1.0 ± 0.06 mmol/L, means ± SEM for 13 nephrotic rats and 11 controls, $P < 0.001$).

In addition to the availability of fatty acids, hepatic triglyceride biosynthesis is influenced by the enzyme activities, including the key enzyme PAP (11). As shown in Table 4, microsomal PAP activity was elevated by 32% in the nephrotic animals, while the cytosolic activity of this enzyme was not affected.

DISCUSSION

A major novel finding in the present work was the increased capacity for hepatic fatty acid oxidation in nephrotic rats. This increase may be mediated through several mechanisms. It has been reported that plasma free fatty acid levels are transiently elevated after the induction of nephrosis (17). Higher plasma free fatty acid levels might result in an increased hepatic fatty acid uptake and initiate adaptive mechanisms to cope with this increased load, i.e., increases in fatty acid oxidation and/or esterification. Thus, increased availability of fatty acids could be a common basis for the increases observed in fatty acid oxidation and triglyceride biosynthesis. Also, the severe hypertriglyceridemia along with a significant accumulation of triglycerides in the liver might trigger compensatory mechanisms, resulting in changes in the partitioning between oxidation and esterification. Whatever the underlying mechanisms, the results obtained are clearly compatible with an increase in fatty acid utilization in nephrotic animals. Furthermore, the degree of increase in fatty acid oxidation was similar utilizing either palmitoyl CoA or palmitoylcarnitine as substrates, arguing against the possibility that the carnitine palmitoyltransferase I (CPT I)-dependent transport of palmitoyl CoA was limiting under these conditions.

In view of the crucial role of carnitine in fatty acid transport through the mitochondrial membrane for subsequent oxidation, it is clearly interesting that hepatic levels of carnitine were increased in nephrotic rats. This finding is consistent with earlier observations that conditions known to stimulate ketogenesis such as starvation and poorly controlled diabetes are associated with increased hepatic carnitine levels (14). However, the nature of this association is unclear. Carnitine is a substrate for CPT I, the enzyme generally believed to be a primary site for the regulation of fatty acid oxidation. Thus, the increase in hepatic carnitine levels might lead to a greater carnitine availability *in vivo* for CPT I and consequently enhanced fatty acid oxidation. Also, there is evidence that carnitine might reduce the sensitivity of CPT I to inhibition by malonyl-CoA which is a major regulator of this enzyme activity (32). It should, however, be pointed out that the increase in the capacity for fatty acid oxidation also can be mediated by effects on other regulatory enzymes in the β-oxidation cycle.

Acetylcarnitine levels in the liver increased in nephrotic rats (Table 2), compatible with increased fatty acid oxidation. It is well-established that the pools of acetylcarnitine and acetyl-CoA are in close relationship, mediated through the action of carnitine acetyltransferase. By this mechanism, intramitochondrially generated acetyl groups bound to CoA can be transferred to carnitine. Acetylcarnitine is then transported across the mitochondrial membrane, making available free CoA intramitochondrially for fatty acid β-oxidation, oxidation of pyruvate, and degradation of branched-chain amino acids (33). Since the pool of tissue carnitine is considerably larger than that of CoA, carnitine can serve as an important acetyl group acceptor (33), a role that has been clearly demonstrated in skeletal muscle during exercise (34,35). In the present study, we were able to demonstrate a statistically significant correlation between acetylcarnitine and acetyl-CoA, indicating that carnitine acts as a buffering substance adjusting the acetyl-CoA/CoA ratio also in the liver. Interestingly, this novel finding was observed in both normal and nephrotic rats.

In nephrotic rats, hepatic triglyceride content as well as VLDL–triglyceride secretion increased, implying enhanced triglyceride biosynthesis in nephrosis. These results are in accordance with the earlier findings that hepatic synthesis of neutral lipids from ^{3}H-palmitate in liver slices from nephrotic rats is increased (8). Interestingly, the increase in VLDL–triglyceride secretion was moderate compared to the substantial accumulation of triglycerides in the liver. These findings are compatible with a compartmentalization of triglycerides into different metabolic pools in the liver. It has been suggested that hepatic triglycerides occur in two pools, a cytoplasmic storage pool and a microsomal secretory pool involved in VLDL formation (9,36,37). However, the interrelationship between the two pools and the mechanisms by which newly synthesized triglycerides are channeled into these pools are poorly understood. Although the present data indicate that both the storage and the secretory pools are increased in nephrosis, a large proportion of newly synthesized triglycerides was apparently retained in the liver.

In nephrotic rats, the increases found in hepatic content and secretion of triglycerides were accompanied by a higher activity of microsomal PAP. PAP, which catalyzes the dephosphorylation of phosphatidic acid to diacylglycerol, is believed to play a prominent role in controlling triglyceride biosynthesis (reviewed in Ref. 38). PAP activity is also present in the cytosol (38,39). It has been suggested that the cytosolic enzyme represents an inactive reserve that is activated when it is translocated onto the endoplasmic reticulum (38). This translocation is promoted by long-chain fatty acids (38). Our observation of an increased microsomal PAP activity suggests that induction of this enzyme activity might contribute to an enhanced triglyceride biosynthesis in nephrosis, and raises the possibility that increased triglyceride biosynthesis may be due to increased availability of the substrate, fatty acids, as well as regulation on the enzyme level. The effects on PAP might, however, be secondary to changes in the availability of fatty acids. Indeed, several hepatic enzyme activities involved in fatty acid synthesis are reported to be elevated, at least transiently after the induction of nephrosis (17), suggesting enhanced fatty acid synthesis. However, primary effects of nephrosis on triglyceride-synthesizing enzymes cannot be excluded. It should be emphasized that there was a discrepancy between the substantial increase in liver triglyceride levels and the slight increase in PAP activity. It seems therefore unlikely that the relatively modest increase in PAP activity in nephrosis can explain the increase in hepatic triglyceride levels. However, as seen from our findings on carnitine and fatty acid oxidation, nephrosis represents a dynamic condition, and an accumulation of triglycerides in the liver after the induction of nephrosis could down-regulate the enzyme activity, thus counteracting more marked initial effects. Such an inhibition of hepatic PAP activity by accumulated lipids has been suggested to occur in chronic alcohol feeding (40).

To summarize, the data presented indicate that the liver capacity for mitochondrial fatty acid oxidation is increased in nephrotic syndrome. This increase might, in part, be mediated through increased carnitine availability. Further, the role of carnitine as an acetyl group acceptor/CoA regenerator in the liver seems to be preserved in nephrotic syndrome in spite of the severe disturbances observed in hepatic lipid metabolism in this condition. The results further suggest that hepatic triglyceride biosynthesis is enhanced in nephrosis, and that this is, in part, mediated through PAP. Moreover, the increase in triglyceride biosynthesis led to a retention of triglycerides in the liver and a disproportionately slight increase in VLDL–triglyceride secretion. These findings suggest that the mechanisms that normally regulate the partitioning of triglycerides between storage and secretion pools are disturbed in the nephrotic syndrome. The precise nature of this disturbance is not known and warrants further studies.

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Effect of Conjugated Linoleic Acid on Body Composition in Mice

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ABSTRACT: The effects of conjugated linoleic acid (CLA) on body composition were investigated. ICR mice were fed a control diet containing 5.5% corn oil or a CLA-supplemented diet (5.0% corn oil plus 0.5% CLA). Mice fed CLA-supplemented diet exhibited 57% and 60% lower body fat and 5% and 14% increased lean body mass relative to controls ($P < 0.05$). Total carnitine palmitoyltransferase activity was increased by dietary CLA supplementation in both fat pad and skeletal muscle; the differences were significant for fat pad of fed mice and skeletal muscle of fasted mice. In cultured 3T3-L1 adipocytes CLA treatment (1×10^{-4} M) significantly reduced heparin-releasable lipoprotein lipase activity (–66%) and the intracellular concentrations of triacylglyceride (–8%) and glycerol (–15%), but significantly increased free glycerol in the culture medium (+22%) compared to control ($P < 0.05$). The effects of CLA on body composition appear to be due in part to reduced fat deposition and increased lipolysis in adipocytes, possibly coupled with enhanced fatty acid oxidation in both muscle cells and adipocytes. *Lipids 32,* 853–858 (1997).

CLA is the acronym for a group of positional and geometric isomers of conjugated dienoic derivatives of linoleic acid. They were identified as comprising an anticarcinogenic principal present in grilled ground beef and other food sources, especially dairy products (1–3). Synthetically prepared CLA inhibited carcinogen-induced neoplasia in several animal models (2,4,5) and reduced development of atherosclerosis in rabbits (6) and hamsters (7). CLA reduced tissue arachidonic acid levels and protected against the catabolic effects of endotoxin administration in mice, rats, and chickens without adversely affecting immune function (8,9). Recently it was established that CLA is a growth factor for young rats in that it enhanced body mass and feed efficiency (10). We now report the effects of CLA on body composition in mice.

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Abbreviations: CLA, conjugated linoleic acid; CPT, carnitine palmitoyltransferase; DMEM, Dulbecco's modified Eagle's medium; LPL, lipoprotein lipase.

METHODS AND PROCEDURES

Materials. Linoleic acid was purchased from Nu-Chek-Prep Corporation (Elysian, MN); triolein, [9,10-^{3}H(N)], (specific activity 12 Ci/mmol) from American Radiolabeled Chemicals Incorporated (St. Louis, MO); and [1-^{14}C]linoleic acid (specific activity 55 mCi/mmol) from Amersham Life Science (Arlington Heights, IL). CLA was prepared as described (1).

Animals and body composition analyses. Weanling male ICR mice (experiment 1), 6-wk-old female (experiment 2) ICR mice, 6-wk-old male ICR mice [carnitine palmitoyltransferase (CPT) assay], feed ingredients (except for CLA), and semi-purified diet (TD94060, 99% basal mix) (Table 1) were purchased from Harlan Sprague-Dawley (Madison, WI). Diet was stored at –20°C until use. Mice were housed in a windowless room with a 12-h light-dark cycle in strict accordance to guidelines established by the Research Animal Resources Center of University of Wisconsin–Madison. Diet was fed *ad libitum*, freshly provided every day (experiment 1) or three times per week (experiment 2). After a 5-d adaptation period mice were randomly separated into groups and fed either control diet (5.5% corn oil) or CLA-containing diet

TABLE 1
Diet Composition[a]

Ingredients	Treatment	
	Control (g/kg)	CLA (g/kg)
Sucrose	481	481
Casein, "Vitamin-Free" Test	210	210
Corn starch	150	150
DL-Methionine	3	3
Corn oil	55	50
CLA	0	5
Cellulose	50	50
Mineral mix, AIN-76	35	35
Vitamin mix, AIN-76A	10	10
Calcium carbonate	4	4
Choline bitartrate	2	2
Ethoxyquin	0.1	0.1

[a]In experiment 2, diet was prepared as described in Reference 37, which contained the same amount of ingredients except 495 g sucrose and 200 g casein per kg diet, without additional calcium carbonate. CLA, conjugated linoleic acid.

(5.0% corn oil plus 0.5% CLA). For body composition analyses, animals were sacrificed, gut contents were removed (to obtain empty carcass weight), and the carcasses frozen at −20°C. Frozen carcasses were chopped, ground, and freeze-dried to determine water content. Total nitrogen was analyzed by the Kjeldahl method (11). Carcass fat content was measured by extraction with diethyl ether overnight using a Soxhlet apparatus. Total ash content was determined by incineration (500–600°C, overnight).

Cell culture. 3T3-L1 preadipocytes were purchased from American Type Culture Collection (Rockville, MD) and cultured as described (12). Briefly, 3T3-L1 preadipocytes were grown to confluence at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. At 2-d post-confluence (designated day 0) cell differentiation was induced with a mixture of methylisobutylxanthin (0.5 mM), dexamethasone (0.25 µM), and insulin (1 µg/mL) in DMEM containing 10% fetal bovine serum. On day 2 this medium was replaced with medium containing 10% fetal bovine serum and insulin only. On day 4 and thereafter the medium consisted of DMEM plus 10% fetal bovine serum only; this medium was subsequently replaced with fresh medium at 2-d intervals.

Fatty acid–albumin complexes were prepared as described (13) with slight modifications and added to culture media 48 h prior to cell harvest. CLA (10 µmol) or linoleic acid (10 µmol), dissolved in 0.1 M KOH, was added to 10 µmol bovine serum albumin solution in phosphate-buffered saline and incubated overnight at 4°C. The pH and volume were adjusted, respectively, to 7.2 and 5.0 mL (before filter sterilization) before use. Final concentrations of linoleic acid and CLA were 100 µM except as indicated for the dose–response experiment. All dishes including control had final concentration of 100 µM albumin.

Cell viability was tested using a number of parameters, as follows. The activities of citrate cleavage enzyme (14) and fatty acid synthetase (15) were determined. In addition 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO) was used as described (16). Briefly, cells were cultured in a 96-well plate and treated with fatty acid–albumin complexes for 48 h as described above. After rinsing, cells were incubated with 50 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL) in DMEM at 37°C for 4 h. Medium was then removed and 200 µL of dimethyl sulfoxide added to each well to dissolve formazan crystals before quantifying at 570 nm with a Microplate Reader Model MR600 (Dynatech Industries, Inc., Alexandria, VA).

Free and esterified glycerol were determined using a Sigma Diagnostic Kit. Heparin-releasable lipoprotein lipase (LPL) activity (E.C. 3.1.1.34, 10 U heparin/mL media for 1 h at 37°C) was measured as described (17). Recovery of free fatty acid was estimated at 71% by using [^{14}C]linoleic acid. Protein was determined as described (18).

CPT (E.C. 2.3.1.23) assay. Mice were fed CLA-supplemented or control diet for 1 wk. Half the mice in each group were then fasted overnight prior to sacrifice by cervical dislocation. Epididymal fat pad, skeletal muscle (hind leg), and liver were removed. Tissues were then homogenized in 3 vol (wt/vol) of 0.25 M sucrose, 1 mM EDTA, 10 mM Tris HCl, pH 7.4 at 4°C. Homogenates were centrifuged (700 × *g* for 10 min); supernatant fluid was again centrifuged (12,000 × *g* for 15 min). Pellets (mitochondrial fraction) were washed and stored at −70°C after resuspension in 70 mM sucrose, 220 mM mannitol, 2 mM HEPES buffer, pH 7.4 and 1 mM EDTA. Total CPT activity was assayed by measuring the initial rates of CoASH formation (19).

Statistical analyses. Data in Figures 1 and 2 and Tables 2 and 3 were analyzed using the "2 independent sample *t*-test." Feed intake per cage was determined every day for experiment 1 (two mice per cage) or at 2–3-d intervals for experiment 2 (one mouse per cage). Feed intake data shown in Figures 1 and 2 are calculated under the assumption that in experiment 1 each mouse acted independently, and in experiment 2 mice consumed an equal amount of feed on each of the days constituting the feeding period. Two-way analysis of variance were performed on data (treatments and experiments) for Figure 3, Table 4, and the carcass protein analyses described in the Results section. Of major interest here are the comparisons among the treatments; these were computed using the Statistics Analysis System (SAS Users' Guide: Statistics, SAS Institute Inc., Cary, NC) with the General Linear Mean procedure and Least Square Means option. If the interaction between treatment and experiment was significant, this interaction was then used as the error term in the Least Square Means analysis.

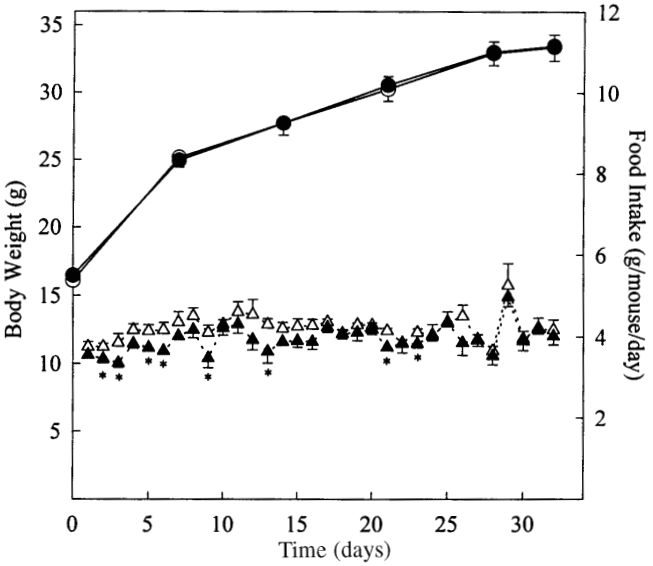

FIG. 1. Body weight (circle) and feed intake (triangle) from male mice fed control or conjugated linoleic acid (CLA)-supplemented diet (experiment 1). Four-week-old male mice were fed either control (open) or CLA (filled)-supplemented diet (0.5% of diet) for 32 d. Body weights ($n = 8$) and feed intake were subjected to *t*-test (*$P < 0.05$). Numbers are mean ± SE.

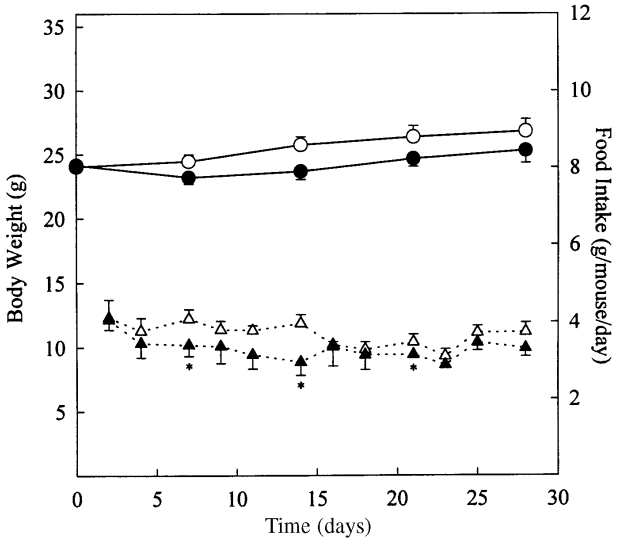

FIG. 2. Body weight (circle) and feed intake (triangle) from female mice fed control or CLA-supplemented diet (experiment 2). Seven-week-old female mice were fed either control (open) or CLA (filled) supplemented diet (0.5% of diet) for 28 d. Body weights and feed intake were subjected to *t*-test (*$P < 0.05$). Numbers are mean ± SE ($n = 6$). See Figure 1 for abbreviation.

RESULTS

Figure 1 shows that male mice fed CLA-supplemented diet exhibited body weights that were indistinguishable from controls. By contrast, the data of Figure 2 indicate that female mice fed CLA appeared to exhibit slightly reduced body weight relative to controls, although the apparent reduction was not statistically significant. In both studies (Figs. 1 and 2) *ad libitum* feed intake for the CLA-fed animals appeared to be reduced at some experimental time points. We have previously shown that small differences in feed intake do not affect body composition (20).

Table 2 shows the effect of CLA supplementation on body composition in these animals. Relative to their respective controls, the percentage body fat in CLA-fed mice was reduced by 57% (males) and 60% (females). Hence, despite similar body weights (Figs. 1 and 2), dietary CLA supplementation resulted in significantly less body fat (Table 2).

By contrast, the percentages of whole body protein and carcass water were significantly enhanced for CLA-fed mice in both experiments (Table 2). Because of the relatively small numbers of animals studied it is not possible to conclude from these data alone that CLA induced a significant increase in whole body protein accretion (whole body protein mass for controls and CLA-fed groups, respectively, were 5.7 and 6.0 g in experiment 1 and 4.4 and 4.6 g in experiment 2; in neither case was the difference significant). However, to date we have conducted 10 such mouse CLA feeding trials, only two of which are reported here in Figures 1 and 2 and Table 2. An analysis of the combined data from these 10 studies indicates that mice fed CLA-supplemented diets do in fact exhibit increased whole body protein relative to controls ($P = 0.0401$).

The mechanism of action of body composition changes is of considerable importance. Accordingly we studied the effect of CLA on total CPT, which is rate-limiting for fatty acid β-oxidation (Table 3). CPT activity was increased by dietary CLA supplementation in both fat pad and skeletal muscle; the differences were significant for fat pad of fed mice, and skeletal muscle of fasted mice. Liver CPT activity was not affected by CLA feeding.

Another key enzyme in lipid metabolism is adipocyte LPL, which hydrolyzes free fatty acids from circulating triacylglyceride; the fatty acids are then taken up by the adipocytes and re-esterified. We utilized 3T3-L1 adipocytes to measure LPL as well as fat mobilization. The amount of CLA added to the cell culture media was based on our determination that the mean CLA content of sera from rats fed diet supplemented with 0.5% CLA for 14 or 28 d is 72 μM (range 23–120 μM).

The data of Figure 3 show that supplementing the culture medium of fully differentiated 3T3-L1 adipocytes with CLA (but not linoleic acid) significantly reduced LPL activity. The inhibitory effect was linear between 20 and 200 μM CLA (Fig. 4). The data indicate a threshold below 20 μM where inhibition is not observed; the apparent enhancement at 5 μM CLA is not understood and requires further study. In separate experiments to determine cell viability we found that incubating 3T3-L1 adipocytes for 2 d with 5 to 200 μM CLA or linoleic acid did not change cell viability as measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (data not shown). We also found that incubating 3T3-L1 adipocytes for 2–3 d with CLA under similar conditions did not reduce citrate cleavage enzyme or fatty acid synthetase ac-

TABLE 2
CLA-Induced Changes in Body Composition[a]

Experiment		ECW (g)[b]	Fat (%)	Protein (%)	Water (%)	Ash (%)
1	Control	32.4 ± 1.1	10.13 ± 1.17	17.76 ± 0.30	66.3 ± 0.8	3.08 ± 0.14
	CLA	32.2 ± 0.8	4.34 ± 0.40[f]	18.58 ± 0.14[d]	70.9 ± 0.4[e]	3.24 ± 0.05[c]
2	Control	25.0 ± 0.9	18.68 ± 3.08	17.67 ± 0.61	58.3 ± 2.2	3.67 ± 0.15
	CLA	23.1 ± 1.0	7.47 ± 0.59[d]	20.09 ± 0.24[d]	66.2 ± 0.7[f]	4.07 ± 0.09

[a]Study duration was 32 d (experiment 1) and 28 d (experiment 2). Reported values are means ± SE ($n = 6$ to 8).
[b]Empty carcass weight.
[c–f]Indicates CLA-fed mice are different from control, where [c]$P < 0.05$, [d]$P < 0.01$, [e]$P < 0.001$, and [f]$P < 0.0001$. For other abbreviation, see Table 1.

TABLE 3
Total Carnitine Palmitoyltransferase Activity in Fat Pad, Muscle, and Liver[a]

	Carnitine palmitoyltransferase (nmol[b]/min/mg protein)		
	Fat pad	Muscle	Liver
Fasted			
Control	15.9 ± 1.7	16.8 ± 2.5	16.7 ± 1.6
CLA	18.4 ± 1.9	25.9 ± 2.1[c]	17.7 ± 1.3
Fed			
Control	14.7 ± 2.1	11.7 ± 1.2	5.3 ± 0.9
CLA	22.4 ± 1.4[c]	14.3 ± 1.7	5.3 ± 0.6

[a]Mice were fed control or CLA-supplemented diet (0.5%) for 1 wk. Reported values are means ± SE (n = 6).
[b]nmols *l*-carnitine exchange (Ref. 23).
[c]$P < 0.05$ compared to control. For abbreviation, see Table 1.

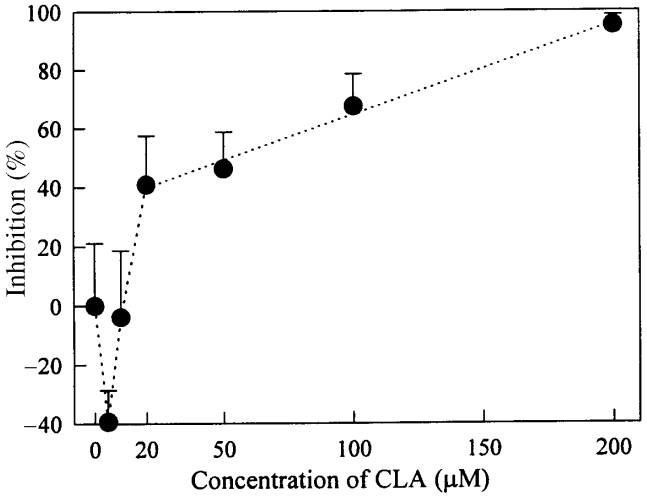

FIG. 4. Effect of CLA dose on LPL activity in 3T3-L1 adipocytes. Reported values are means ± SE of 3–4 culture dishes normalized against control. The r^2 is 0.9901 for the data between 20 and 200 μM CLA. For abbreviations, see Figures 1 and 3.

tivities (data not shown). Finally, there were no CLA- or linoleic acid-induced differences in total protein or heparin-released protein from the cells. Hence, the CLA effect reported here appears to be specific and not due to toxicity.

Table 4 shows the amount of esterified and free glycerol within the cells, and the free glycerol in the culture medium. The findings are consistent with Figures 3 and 4 in that CLA treatment resulted in a significant reduction in intracellular triacylglyceride (determined as esterified glycerol). However, the significant increase in free glycerol in the media indicates that CLA may have stimulated lipolysis as well. Linoleic acid also appears to have stimulated lipolysis in these cells, which is consistent with the findings of Takada *et al.* (21) that body fat was modestly reduced in rats fed gamma-linolenic acid (a metabolite of linoleic acid).

DISCUSSION

The data of Table 2 establish that mice fed CLA-supplemented diets exhibited significant changes in body composition relative to controls. This observation should be considered with regard to effects on the adipocyte (the principal site of fat storage) and the skeletal muscle cell (the principal site of fat oxidation).

The study described in Table 3 was conducted with tissues from control and CLA-fed mice. The findings are consistent with the interpretation that feeding mice a diet supplemented with CLA enhances fatty acid β-oxidation in skeletal muscle and fat pad, but not liver. These data (Table 3) may also explain at least in part our previous report (6) that rabbits fed CLA-supplemented diet exhibited reduced serum triglyceride levels.

The experiments described in Figures 3 and 4 and Table 4, which were conducted with 3T3-L1 adipocytes, indicate that CLA treatment reduced LPL activity while apparently enhancing lipolysis. If we assume that the *in vitro* experiments

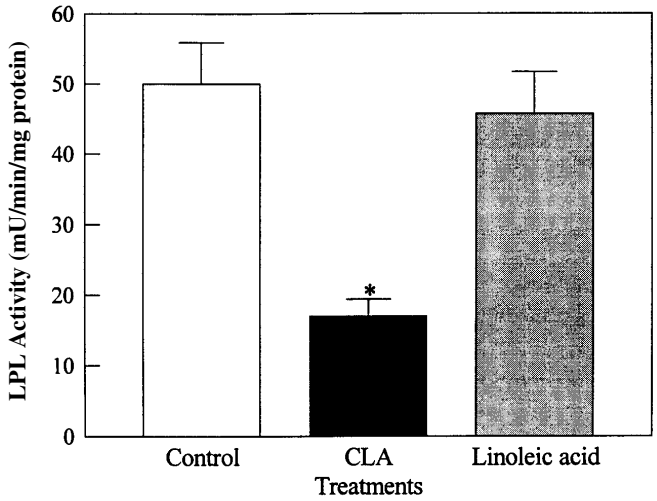

FIG. 3. CLA inhibits lipoprotein lipase (LPL) activity in 3T3-L1 adipocytes. Reported values are means ± SE (n = 15–17, collected from four independent experiments). *$P < 0.05$. See Figure 1 for abbreviation.

TABLE 4
Esterified and Free Glycerol in Cell Sonicates, and Free Glycerol in Culture Medium from Control and CLA-Treated 3T3-L1 Adipocytes[a]

	Percentage of control		
	Control	CLA	Linoleic acid
Esterified glycerol in cell	100[a]	91.7± 6.4[b]	117.1 ± 8.0[c]
Free glycerol in cell	100[a]	84.9 ± 7.2[b]	107.4 ± 5.9[a]
Free glycerol in culture medium	100[a]	122.0 ± 12.9[b]	106.6 ± 2.2[b]

[a]Cells were treated with 100 μM of CLA or linoleic acid for 48 h before assay. The means are for 10 independent experiments, normalized against their respective controls. Data (2–6 dishes per treatment in each experiment) were analyzed as log value with a two-way analysis of variance using fatty acid (as treatments) and experiment. If the interaction between treatment and experiment was significant, this interaction was used as the error term. Means with different superscript letters are significantly different ($P < 0.05$). For abbreviation, see Table 1.

with 3T3-L1 adipocytes (Figs. 3, 4, Table 4) reflect *in vivo* physiological changes, then these data taken together with the data of Table 3 appear to provide a framework for partially understanding the reduction in body fat that is evident in the data of Table 2. It should be noted that the data of Figures 3 and 4 and Table 4 support our very recent findings (22) that adipocytes isolated from rats fed CLA exhibited increased hormone-sensitive lipase activity and enhanced norepinephrine-induced lipolysis.

A possible explanation for the apparent enhancement of lean body mass (Table 2 and accompanying discussion of whole body protein accretion in the Results section) is less clear. This may be related to our previous reports that CLA protects against the catabolic effects of immune stimulation (8,9), which is modulated by prostaglandin E_2 and mediated by interleukin-1 and tumor necrosis factor-α, both of which are linked to obesity (23–26). It is also possible that CLA affects pathways induced by anabolic hormones, perhaps in conjunction with an adaptive response by skeletal muscle necessitated by increased lipid substrate for β-oxidation. Further investigation is required.

There appear to be parallels between the effects of CLA reported and discussed herein and the reported effects of dietary fish oil. Like CLA, fish oil is reported to reduce fat pad size (27, but also see 28), prevent cachexia and body weight loss following immune stimulation (29,30), and reduce tissue arachidonic acid levels (31,32). The omega-3 fatty acids of fish oil modulate numerous biological and physiological effects that are linked to tumor necrosis factor-α, interleukin-1, and the eicosanoid pathways (30,33), indicating that the effects of CLA may also involve eicosanoid mechanism(s).

The isomers of CLA may be metabolized to one or more biologically active products which exert biological effect. For example CLA-derived eicosanoids could regulate the synthesis of arachidonic acid metabolites like prostaglandin E_2. Alternatively, CLA-derived eicosanoids might exhibit biological activity in their own right. These possibilities are at this time speculation, but they are also the focus of considerable ongoing research (8,9,34–36). Understanding the biochemical mechanism(s) of action of CLA will be crucial to fully utilizing the potential of this class of fatty acids in animal husbandry and human application.

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Correlation of Suppressed Linoleic Acid Metabolism with the Hypocholesterolemic Action of Eritadenine in Rats

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ABSTRACT: The dose-dependent effects of dietary eritadenine on the metabolism of linoleic acid and on the plasma cholesterol concentration were investigated to clarify the mechanism of the hypocholesterolemic action of eritadenine in rats. Rats were fed control or eritadenine-supplemented (2 to 20 mg/kg) diets for 14 d. Eritadenine supplementation significantly decreased both the plasma cholesterol concentration and the 20:4n-6/18:2n-6 ratio of liver microsomal and plasma phosphatidylcholine (PC) in a dose-dependent manner. Eritadenine was also found to decrease the activity of Δ6 desaturase in liver microsomes; there was significant correlation between the Δ6–desaturase activity and the 20:4n-6/18:2n-6 ratio in the PC of liver microsomes ($r = 0.989$, $P < 0.001$) or plasma ($r = 0.986$, $P < 0.001$). Certain plasma PC molecular species, as represented by 16:0-18:2, were increased by eritadenine in a dose-dependent manner, and certain plasma PC molecular species, as represented by 18:0-20:4, were conversely decreased by eritadenine. There was a significant correlation between the plasma total cholesterol concentration and the proportion of the sum of plasma PC molecular species which contain 18:1 or 18:2 in the *sn*-2 position. These results support the idea that the suppression of linoleic acid metabolism by eritadenine might be associated with the hypocholesterolemic action of eritadenine.
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Eritadenine [2(*R*),3(*R*)-dihydroxy- 4-(9-adenyl)-butyric acid] is known to be a hypocholesterolemic factor of the edible mushroom *Lentinus edodes* (Shiitake in Japanese) (1,2). The mechanism underlying the hypocholesterolemic action of the compound is not yet fully elucidated. Recently, we found that eritadenine decreases the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) in rat liver microsomes through the depression of PE *N*-methylation (3,4), and that the compound suppresses the metabolism of linoleic acid into arachidonic acid, thereby leading to modifications of liver microsomal and plasma PC molecular species (5,6). It is reasonable to deduce that the decreased PC/PE ratio of liver microsomes may be associated with the hypocholesterolemic action of eritadenine, since there was a significant correlation between the PC/PE ratio and plasma cholesterol concentrations (3,4). However, it is still unclear whether the suppression of linoleic acid metabolism also participates in the hypocholesterolemic action of eritadenine. The mechanism of the suppression of linoleic acid metabolism by eritadenine is also unclear.

In this study, therefore, the dose-dependent effects of dietary eritadenine on various variables concerning lipid metabolism were investigated to clarify the relationship between the suppression of linoleic acid metabolism and the hypocholesterolemic action of eritadenine in rats.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Linoleic acid (1.7 GBq/mmol) was obtained from New England Nuclear (Boston, MA). Unlabeled linoleic acid and other biochemicals were provided by Sigma (St. Louis, MO) and Wako Pure Chemical (Osaka, Japan). All other chemicals were purchased from Wako and were of analytical grade. Eritadenine was isolated from dried *L. edodes* mushroom according to the method of Tokita *et al.* (7), and the purity of eritadenine used was at least 95% when assayed by thin-layer chromatography (TLC) (8).

Animals. Forty-eight male 5-wk-old rats of the Wistar strain were received at 90–100 g body weight from Japan SLC (Hamamatsu, Japan). The rats were individually housed in hanging stainless-steel wire cages kept in an isolated room at a controlled temperature (23–25°C) and humidity (40–60%). Lights were maintained on a 12-h cycle (light on from 0600 to 1800 h). For 5 or 6 d, rats were fed the powdered laboratory stock diet, which resembled the control diet described below except that sucrose and lactose were replaced by starch, and 2 g/kg diet of choline chloride was added. Then they were divided into six groups of eight rats each with similar mean weights (~123 g) and allowed free access to the experimental diets and water for 14 d. The experimental plan was approved by the Laboratory Animal Care Committee of the Faculty of Agriculture, Shizuoka University (Shizuoka, Japan).

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Abbreviations: GLC, gas–liquid chromatography; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

Diets. Six diets, control and eritadenine-supplemented (five levels) diets, were utilized in this study. The control diet consisted of the following (g/kg): casein (Nacalai tesque, Kyoto, Japan), 250; corn starch (Fuji-seifun, Shimizu, Japan), 450; sucrose (Fuji-seito, Shimizu, Japan), 200; corn oil (Honen, Tokyo, Japan), 50; AIN-76 mineral mixture (Oriental Yeast, Tokyo, Japan), 35; AIN-76 vitamin mixture (Oriental), 10; choline chloride (Wako), 4; and lactose (Wako), 1. Eritadenine was fully mixed with lactose and added to the diet at levels of 2, 4, 6, 10, and 20 mg/kg diet at the expense of lactose. The fatty acid composition of corn oil used was as follows (wt%): 16:0, 12.2; 18:0, 1.8; 18:1, 32.8; 18:2n-6, 51.4; and 18:3n-3, 1.6.

Tissue collection and fractionation. At the end of the feeding period, nonfasted rats were killed by decapitation under light anesthesia with diethyl ether between 1100 and 1200 h. Plasma was separated from heparinized whole blood by centrifugation at 2000 × *g* for 20 min at 4°C. An aliquot of the plasma was stored at 4°C until subsequent analyses for plasma lipid concentrations, and the residual plasma was stored at −80°C until analyses for phospholipids. After collection of blood, the whole liver was quickly removed, rinsed in ice-cold saline, blotted on filter paper, and weighed. The liver was homogenized in four volumes (vol/wt) of an ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.15 M KCl, a modified solution of Nguyen *et al.* (9). An aliquot (12 mL) of the homogenate was centrifuged at 10,000 × *g* for 12 min at 4°C, and the resultant supernatants were further centrifuged at 105,000 × *g* for 60 min at 4°C to obtain the microsomal fraction as a precipitate. The microsomes obtained were resuspended in the homogenizing buffer and stored at −80°C until analyses for phospholipids and enzyme activity. Another aliquot (2 mL) of the liver homogenate was stored at −30°C until analyses for liver lipid concentrations.

Lipid analysis. The plasma concentrations of total cholesterol, high density lipoprotein (HDL) cholesterol, free cholesterol, triglycerides, and phospholipids were measured enzymatically with kits (Cholesterol C-Test, HDL Cholesterol-Test, Free Cholesterol C-Test, Triglyceride G-Test, and Phospholipid B-Test, respectively; Wako). The difference between total cholesterol and HDL cholesterol or free cholesterol was assumed to be very low density lipoprotein (VLDL) + low density lipoprotein (LDL) cholesterol or esterified cholesterol, respectively. The total lipids of liver homogenate, liver microsomes, and plasma were extracted by the method of Folch *et al.* (10). The cholesterol, triglycerides, and phospholipids in the extract of liver homogenate were measured according to Zak (11), Fletcher (12), and Bartlett (13), respectively. For the determination of phospholipid class composition, the phospholipids in the extract of liver microsomes were separated into each class by TLC on silica gel 60 (E. Merck, Darmstadt, Germany) using chloroform/methanol/water (65:25:4, by vol) as a developing solvent. The bands of each phospholipid class were visualized in iodine vapor, scraped off the plate, and analyzed directly for inorganic phosphorus (13). For the determinations of fatty acid and molecular species composition, PC was likewise separated by TLC from liver microsomes and plasma, visualized with dichlorofluorescein, scraped off the plate, and extracted with chloroform/methanol (1:2, vol/vol). An aliquot of PC was treated with 14% (w/w) BF_3/methanol reagent (Wako), and fatty acid methyl esters formed were analyzed by gas–liquid chromatography (GLC) on a Model GC-17A (Shimadzu, Kyoto, Japan), equipped with a TC-FFAP capillary column (0.25 mm × 30 m; GL Sciences, Tokyo, Japan). Another aliquot of PC was converted to diacylglycerol benzoates according to the method of Blank *et al.* (14). The diacylglycerol benzoates were analyzed by high-performance liquid chromatography (HPLC) on a Model LC-6A system (Shimadzu), equipped with an ODS column (4.6 × 250 mm, Lichrosorb RP-18; E. Merck), essentially according to the method of Blank *et al.* (14). Since some peaks consisted of two molecular species, the ratio of two molecular species was determined by GLC. A representative chromatogram of PC molecular species by HPLC was shown previously (5).

Enzyme assay. The activity of Δ6 desaturase in liver microsomes was measured essentially according to the method of Svensson (15). The reaction mixture (total 1.5 mL) consisted of 75 mM phosphate buffer (pH 7.0), 0.15 M KCl, 0.25 M sucrose, 0.5 mM nicotinamide, 5.5 mM $MgCl_2$, 5 mM ATP, 1.5 mM glutathione, 0.5 mM CoA, 1 mM NADH, 67 μM [1-^{14}C]linoleic acid (56 MBq/mmol), and liver microsomes (*ca.* 3 mg). After preincubation for 10 min at 37°C, the reaction was started by adding NADH, and the reaction mixture was further incubated for 20 min at 37°C in a test tube of wide bore (16 mm) under reciprocal shaking (120 cycle/min). The reaction was stopped by adding 1 mL of methanol containing 10% KOH (wt/vol). After saponification and subsequent acidification, fatty acids were extracted with petroleum ether. The extracted fatty acids, together with carrier fatty acids (linoleic acid and γ-linolenic acid), were methylated with BF_3/methanol reagent. The fatty acid methyl esters were separated by HPLC. The effluents of the peaks of γ-linolenic acid and linoleic acid were collected, and the radioactivity was counted by liquid scintillation counter. Protein was measured according to Lowry *et al.* (16) using bovine serum albumin as a standard.

Statistical analysis. Data were expressed as mean ± SEM for eight rats. Data were analyzed by one-way analysis of variance, and the differences between means were tested using Duncan's multiple range test (17) when the *F* value was significant. Simple correlation between variables was calculated by linear regression analysis using mean values. A *P* value of 0.05 or less was considered significant.

RESULTS

Effects on growth, food intake, liver weight, and liver lipid concentrations. There was no significant difference in the body weight gain and food intake of animals among the six groups (Table 1). Relative liver weights were not affected by eritadenine supplementation except for the highest dose. Eritadenine did not affect the concentration of cholesterol in the liver. Eritadenine tended to decrease the hepatic concentra-

TABLE 1
Growth, Food Intake, Liver Weight, and Liver Lipid Concentrations In Rats Fed Graded Levels of Eritadenine[a]

	Dietary eritadenine (mg/kg)					
	0	2	4	6	10	20
Body weight gain (g/14 d)	73 ± 2	75 ± 2	74 ± 2	71 ± 3	77 ± 2	75 ± 3
Food intake (g/14 d)	184 ± 6	186 ± 3	184 ± 4	175 ± 3	185 ± 4	179 ± 4
Liver wt (g/100 g body weight)	4.65 ± 0.08^{b}	4.62 ± 0.13^{b,c}	4.48 ± 0.09^{b,c}	4.39 ± 0.10^{b,c}	4.48 ± 0.07^{b,c}	4.35 ± 0.07^{c}
Liver lipids (μmol/g liver)						
Total cholesterol	11.6 ± 0.1	11.9 ± 0.2	11.4 ± 0.2	11.4 ± 0.2	11.3 ± 0.2	11.8 ± 0.2
Triglycerides	26.8 ± 1.2^{b}	24.4 ± 1.1^{b,c}	20.6 ± 2.4^{c,d}	14.8 ± 0.8^{e}	16.0 ± 0.7^{d,e}	19.3 ± 1.7^{d}
Phospholipids	37.4 ± 0.7^{d}	37.7 ± 0.7^{d}	39.9 ± 0.7^{c}	42.1 ± 0.7^{b}	41.9 ± 0.6^{b}	43.1 ± 0.6^{b}

[a]Values are mean ± SEM for eight rats. Values in a row with different superscript letters (b–e) are significantly different at $P < 0.05$.

tion of triglycerides and inversely to increase the concentration of phospholipids in a dose-dependent manner.

Effects on plasma lipids and liver microsomal phospholipids. Figure 1 shows the effects of dietary eritadenine on plasma lipid concentrations. The plasma total cholesterol concentration was significantly decreased by eritadenine supplementation in a dose-dependent manner, accompanying decreases in VLDL + LDL cholesterol, HDL cholesterol, and esterified cholesterol. The plasma phospholipid concentration was also significantly decreased by eritadenine in a dose-dependent manner, whereas the plasma triglyceride concentration was unaffected by eritadenine. As a whole, the maximal effect of eritadenine on plasma lipids was attained at an eritadenine level of 6 mg/kg diet.

Figure 2 shows the effects of dietary eritadenine on the concentration and proportion of phospholipids in liver microsomes. The concentration of PC was not affected by eritadenine supplementation, whereas the concentration of PE increased in response to eritadenine and reached a plateau at an eritadenine level of 10 mg/kg diet. The concentration of total phospholipids was slightly, but significantly, increased by eritadenine supplementation. The proportion of PC or PE to the total phospholipids was decreased or increased, respectively, by eritadenine supplementation in a dose-dependent manner. Consequently, the PC/PE ratio was also decreased by eritadenine in a dose-dependent manner.

Effects on fatty acid and molecular species composition of PC. Figure 3 shows the effects of dietary eritadenine on the proportion of selected fatty acids (linoleic and arachidonic acid) and their ratio in liver microsomal and plasma PC. The proportion of linoleic acid in liver microsomal PC was significantly increased by eritadenine supplementation in a dose-dependent manner, whereas the proportion of arachidonic acid was significantly decreased by eritadenine. Consequently, the ratio of arachidonic to linoleic acid was significantly decreased by eritadenine in a dose-dependent manner. The effects of eritadenine on linoleic and arachidonic acid in plasma PC were quite similar to those in liver microsomal PC. There was a significantly positive correlation in the ratio of 20:4n-6/18:2n-6 between liver microsomal PC and plasma PC ($r = 0.999$, $P < 0.001$). Furthermore, there was a significantly positive correlation between the 20:4n-6/18:2n-6 ratio and plasma total cholesterol concentrations ($r = 0.990$, $P < 0.001$). The minimal dose of eritadenine to bring about the maximal effect was 10 (for linoleic and arachidonic acid) or 6 (for the ratio of arachidonic to linoleic acid) mg/kg diet. The effects of eritadenine on the other fatty acids of PC were smaller than those on these two fatty acids (data not shown).

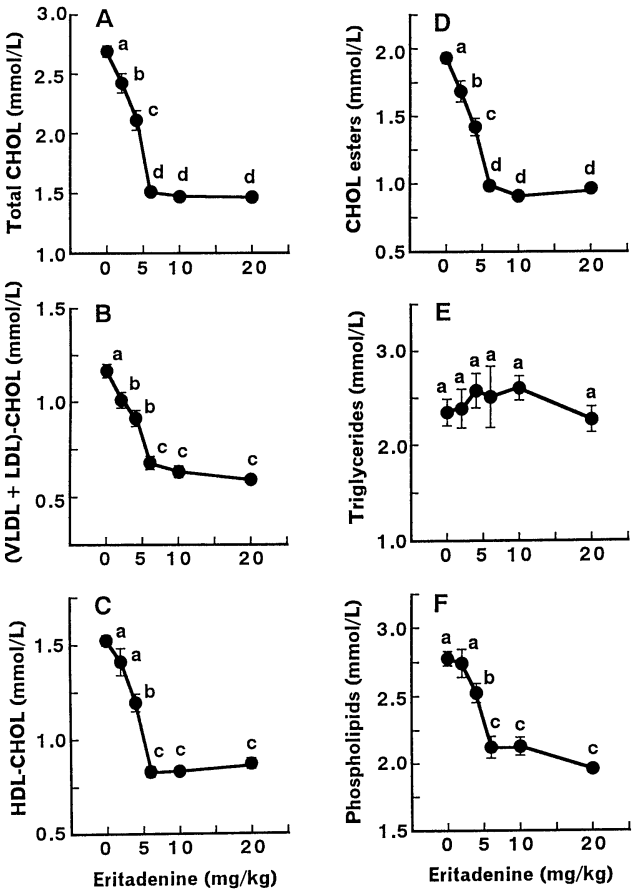

FIG. 1. Dose-dependent effects of dietary eritadenine on the plasma concentration of total cholesterol (A), VLDL + LDL cholesterol (B), HDL cholesterol (C), cholesterol esters (D), triglycerides (E), and phospholipids (F) in rats. The circle and its bar represent mean and SEM, respectively, for eight rats. Values with different letters are significantly different at $P < 0.05$. Abbreviations: CHOL, cholesterol; VLDL, very low density lipoprotein; LDL, low density lipoprotein.

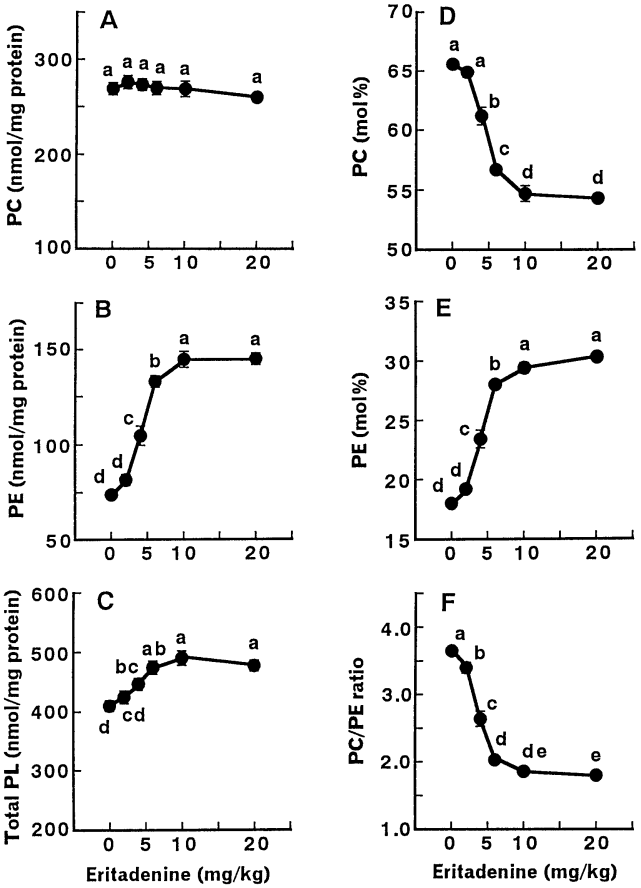

FIG. 2. Dose-dependent effects of dietary eritadenine on the concentration of phosphatidylcholine (A), phosphatidylethanolamine (B), and total phospholipids (C), the proportion of phosphatidylcholine (D) and phosphatidylethanolamine (E), and on the ratio of phosphatidylcholine to phosphatidylethanolamine (F) in liver microsomes of rats. The circle and its bar represent mean and SEM, respectively, for eight rats. Values with different letters are significantly different at $P < 0.05$. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids.

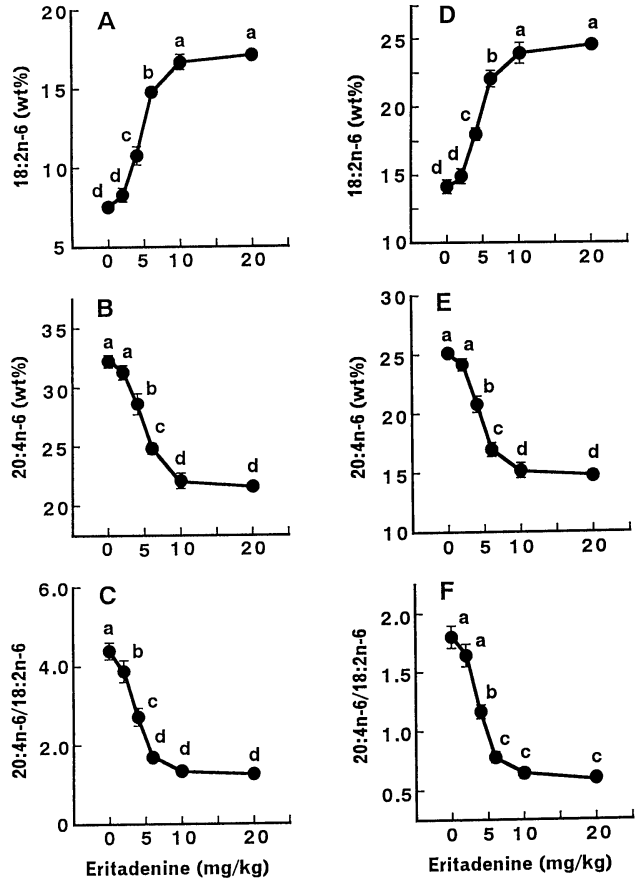

FIG. 3. Dose-dependent effects of dietary eritadenine on the proportion of linoleic acid, arachidonic acid, and the ratio of arachidonic to linoleic acid in liver microsomal phosphatidylcholine (A,B,C) and plasma phosphatidylcholine (D,E,F). The circle and its bar represent mean and SEM, respectively, for eight rats. Values with different letters are significantly different at $P < 0.05$.

Table 2 shows the effect of eritadenine on the molecular species composition of plasma PC. The proportion of 16:0-18:1 or three molecular species containing 18:2 in the *sn*-2 position increased in response to eritadenine supplementation in a dose-dependent manner and had a significantly negative correlation with plasma total cholesterol concentrations. On the contrary, the proportion of each molecular species containing 20:4, 22:5, or 22:6 decreased in response to eritadenine supplementation and had a significantly positive correlation with plasma total cholesterol concentrations. Of three PC molecular species containing 18:2 in the *sn*-2 position, the extent of increase in 16:0-18:2 was apparently greater than that of increase in the other molecular species. In contrast, the extent of decrease in 18:0-20:4 was greater than that of increase in 16:0-20:4 or 18:1-20:4. Similarly, eritadenine affected the molecular species composition of liver microsomal PC (data not shown); there were significantly positive correlations in the proportion of 16:0-18:2 or 18:0-20:4 between liver microsomal PC and plasma PC ($r = 0.996$, $P < 0.001$; $r = 0.998$, $P < 0.001$, respectively). There was a significantly negative correlation between the plasma total cholesterol concentration and the sum of the proportion of plasma PC molecular species containing 18:1 or 18:2 in the *sn*-2 position (Fig. 4).

Effect on Δ6-desaturase activity. Figure 5 shows the effect of dietary eritadenine on the activity of Δ6 desaturase in liver microsomes. The enzyme activity was found to be largely decreased by eritadenine supplementation in a dose-dependent manner. At the highest dose level (20 mg/kg), the enzyme activity was decreased to 28.8% of the value of eritadenine-unsupplemented control group. There was a significantly positive correlation between the Δ6-desaturase activity and the 20:4n-6/18:2n-6 ratio in liver microsomal PC ($r = 0.989$, $P < 0.001$). Further, the activity of Δ6 desaturase had a significantly positive correlation with the PC/PE ratio of liver microsomes (Fig. 6). In addition, there were correlations between the enzyme activity and the concentration (nmol/mg protein) of PE ($r = -0.977$, $P < 0.001$), the proportion (mol%)

TABLE 2
Plasma Phosphatidylcholine Molecular Species Composition in Rats Fed Graded Levels of Eritadenine[a]

Molecular species	Dietary eritadenine (mg/kg)						
	0	2	4	6	10	20	r[b]
16:0-18:1[c]	5.8 ± 0.1[g]	6.1 ± 0.2[g]	7.2 ± 0.1[f]	7.9 ± 0.2[e]	8.4 ± 0.2[d]	8.3 ± 0.1[d]	–0.984***
18:0-18:1	1.9 ± 0.1	2.0 ± 0.0	2.0 ± 0.0	1.9 ± 0.1	2.0 ± 0.0	1.9 ± 0.0	–0.117
18:1-18:1	1.3 ± 0.0[e]	1.1 ± 0.2[e,f]	1.0 ± 0.1[f]	1.7 ± 0.1[d]	1.8 ± 0.1[d]	1.8 ± 0.0[d]	–0.805
16:0-18:2	15.5 ± 0.6[h]	16.7 ± 0.6[h]	21.0 ± 0.8[g]	26.1 ± 0.7[f]	28.4 ± 0.8[e]	30.4 ± 0.4[e]	–0.977***
18:0-18:2	11.2 ± 0.2[g]	11.9 ± 0.3[g]	14.1 ± 0.3[f]	15.3 ± 0.4[e]	16.3 ± 0.3[d]	16.1 ± 0.1[d]	–0.984***
18:1-18:2	4.3 ± 0.1[f]	4.4 ± 0.1[f]	4.8 ± 0.1[e]	5.7 ± 0.2[d]	5.9 ± 0.1[d]	5.7 ± 0.1[d]	–0.986***
16:0-20:4	16.7 ± 0.2[d]	16.4 ± 0.3[d]	14.8 ± 0.3[e]	13.4 ± 0.4[f]	12.6 ± 0.3[f]	13.0 ± 0.2[f]	0.985***
18:0-20:4	23.3 ± 0.8[d]	21.8 ± 0.9[d]	18.4 ± 0.8[e]	12.9 ± 0.7[f]	11.1 ± 0.6[fg]	10.2 ± 0.2[g]	0.990***
18:1-20:4	4.4 ± 0.1[de]	4.7 ± 0.3[d]	3.9 ± 0.2[ef]	3.8 ± 0.2[f]	3.1 ± 0.2[g]	3.2 ± 0.2[g]	0.898*
16:0-22:5	2.9 ± 0.1[d]	2.4 ± 0.1[e]	1.7 ± 0.1[f]	1.1 ± 0.0[g]	0.8 ± 0.0[h]	0.5 ± 0.0[i]	0.987***
18:0-22:5	2.7 ± 0.1[d]	2.3 ± 0.1[e]	1.8 ± 0.1[f]	1.1 ± 0.1[g]	0.9 ± 0.0[g]	0.6 ± 0.0[h]	0.987***
16:0-22:6	4.5 ± 0.1[d]	4.4 ± 0.1[d]	4.0 ± 0.1[e]	3.3 ± 0.1[f]	2.7 ± 0.1[g]	2.5 ± 0.1[g]	0.949**
18:0-22:6	2.0 ± 0.0[d]	2.0 ± 0.0[d]	1.7 ± 0.0[e]	1.4 ± 0.1[f]	1.2 ± 0.0[g]	1.1 ± 0.0[h]	0.962**

[a]Values are mean ± SEM for eight rats in mol%. [b]Correlation coefficient with plasma total cholesterol concentration. [c]Fatty acids in the *sn*-1 and *sn*-2 positions of phosphatidylcholine are indicated at the left and right side, respectively. Values in a row with different superscript letters (d–i) are significantly different at $P < 0.05$. *,**,***Significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

of PE ($r = -0.979$, $P < 0.001$), or the proportion of PC ($r = 0.968$, $P < 0.01$) in liver microsomes. However, there was no significant correlation between the enzyme activity and PC concentration in liver microsomes ($r = 0.515$, $P > 0.1$).

DISCUSSION

Effect of eritadenine on linoleic acid metabolism. The present study demonstrated that dietary eritadenine increases the proportion of linoleic acid and conversely decreases the proportion of arachidonic acid in liver microsomal and plasma PC in a dose-dependent manner (Fig. 3). These results can be taken to indicate that eritadenine suppresses the metabolic conversion of linoleic acid into arachidonic acid in rats. In support of this, the activity of Δ6 desaturase in liver microsomes, the rate-limiting enzyme in the metabolism of linoleic acid (18), was found to be largely depressed by eritadenine in a dose-dependent manner (Fig. 5). Furthermore, a significantly positive correlation could be detected between the Δ6-desaturase activity and the 20:4n-6/18:2n-6 ratio in liver microsomal PC. Thus, it is reasonable to conclude that eritadenine suppresses the metabolism of linoleic acid mainly by decreasing the activity of Δ6 desaturase. Leikin and Brenner (19–21) have shown that the activity of Δ6 or Δ5 desaturase was influenced by the composition of liver microsomal membrane constituents, such as PC/PE ratio or PC/cholesterol ratio, in rats. She *et al.* (22) also have shown that there was a significant correlation between the concentration of PC and the activity of Δ6 desaturase in rat liver microsomes which

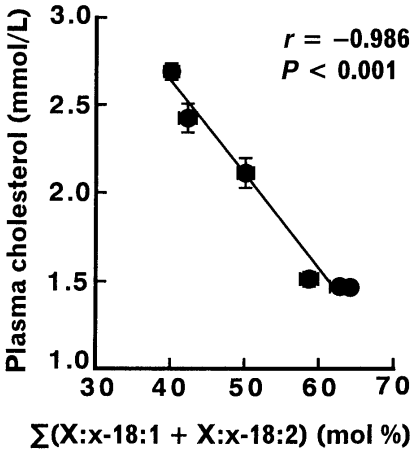

FIG. 4. Correlation between the plasma total cholesterol concentration and the proportion of certain plasma phosphatidylcholine molecular species in rats fed six experimental diets. The circle and its bar represent mean and SEM, respectively, for eight rats. Abbreviation: ∑(X:x-18:1 + X:x-18:2), the sum of phosphatidylcholine molecular species which contain 18:1 or 18:2 in the *sn*-2 position, where X:x denotes fatty acids in the *sn*-1 position (i.e., 16:0, 18:0, or 18:1).

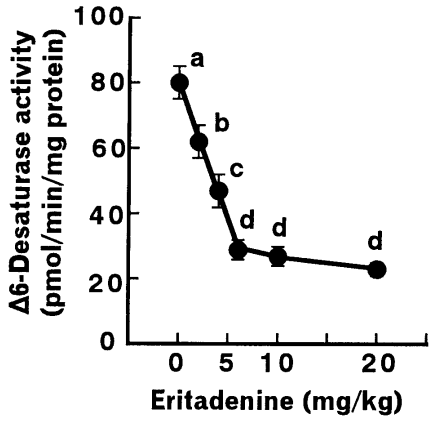

FIG. 5. Dose-dependent effect of dietary eritadenine on the activity of Δ6 desaturase in liver microsomes of rats. The circle and its bar represent mean and SEM, respectively, for eight rats. Values with different letters are significantly different at $P < 0.05$.

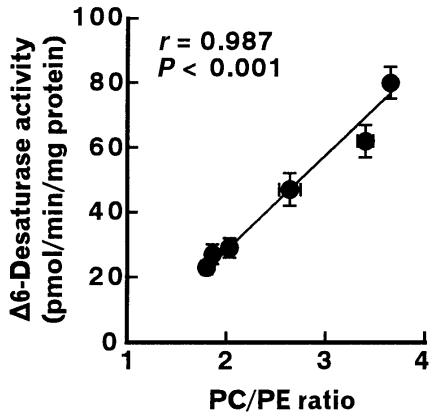

FIG. 6. Correlation between the activity of Δ6 desaturase and the ratio of phosphatidylcholine to phosphatidylethanolamine in liver microsomes of rats fed six experimental diets. The circle and its bar represent mean and SEM, respectively, for eight rats. See Figure 2 for abbreviations.

were treated with phospholipase C *in vitro* to hydrolyze membrane phospholipids for different times. Furthermore, Leikin and Shinitzky (23) have demonstrated that the Δ6 desaturase, which was isolated from rat liver microsomes by high hydrostatic pressure, has an associated lipid surrounding of PC and cholesterol at an approximately 4:1 mole ratio. These findings suggest that the activity of Δ6 desaturase in liver microsomes can be regulated by microsomal phospholipid composition. One of the most striking effects of eritadenine on microsomal phospholipids is an increase in PE concentration and a resultant decrease in the PC/PE ratio (Fig. 2). Therefore, it is reasonable to deduce that eritadenine may decrease the Δ6-desaturase activity, at least in part, through alteration of liver microsomal phospholipid profile. Indeed, the present study could demonstrate that Δ6-desaturase activity correlates with the PC/PE ratio (Fig. 6) or other variables, but not with PC concentration. These results suggest that the increase in PE or the resultant decrease in the proportion of PC, rather than PC concentration itself, might be associated with the eritadenine-induced decrease in Δ6-desaturase activity. At present, it is unclear whether the decrease in Δ6-desaturase activity is accompanied by a decrease in the enzyme mass.

The modification of fatty acid profile in liver microsomal PC caused by eritadenine is considered to reflect the fatty acid profile of plasma PC mainly through the secretion of lipoproteins from the liver into the blood circulation, although plasma PC can be metabolized by lecithin:cholesterol acyltransferase. In support of this, there was a highly significant correlation in the ratio of 20:4n-6/18:2n-6 between liver microsomal PC and plasma PC. This was also the case for the PC molecular species profile. Thus, it is likely that the alteration of liver microsomal phospholipid class profile, as represented by a decrease in the PC/PE ratio, is a cause of the suppressed activity of Δ6 desaturase and thereby finally modifies the fatty acid and molecular species profiles of plasma PC in rats fed eritadenine.

Relationship between linoleic acid metabolism and plasma cholesterol. Earlier reports have shown that eritadenine neither inhibits cholesterogenesis in the liver of rats (24–26) nor stimulates steroid excretion into feces (27). It has also been shown that the clearance rate of plasma cholesterol was faster in eritadenine-treated rats than in control rats (24–26). On the other hand, a slight suppression of hepatic secretion of plasma lipoproteins by eritadenine was observed (28). Eritadenine increases the requirement for dietary choline, since it depresses PC biosynthesis *via* PE *N*-methylation pathway (4). Hence, depressed lipoprotein secretion from the liver and resultant fat accumulation in the liver due to PC deficiency are brought about when adequate amounts of choline are not included in the diet. When the diet contained an adequate amount of choline chloride (8 g/kg diet), the secretion of triglycerides and cholesterol in a form of VLDL, as measured after Triton WR-1339 injection, was not impaired in eritadenine-fed rats compared with control rats (Sugiyama, K., and Yamakawa, A., unpublished data). This suggests that the essential hypocholesterolemic action of eritadenine cannot be ascribed to the depressed secretion of triglyceride-rich lipoproteins from the liver. In support of this, Vance *et al.* (29) have shown that 3-deazaadenosine, another adenosine analog which can depress PC biosynthesis *via* the PE *N*-methylation pathway, did not affect the secretion of apoproteins and PC from cultured rat hepatocytes even though the cellular PC/PE ratio was significantly decreased. Taken together, these results suggest that eritadenine may exert its hypocholesterolemic action mainly through the enhanced uptake of plasma lipoprotein cholesterol by tissues. In addition to the hypocholesterolemic action, the increase in linoleic acid or the decrease in arachidonic acid due to the suppression of linoleic acid metabolism is another noticeable metabolic change caused by eritadenine. Therefore, it seems reasonable to consider that the suppression of linoleic acid metabolism also may be associated with the hypocholesterolemic action of eritadenine. Actually, in the present study, there was a significant correlation between the 20:4n-6/18:2n-6 ratio and plasma total cholesterol concentrations. It is established that dietary linoleic acid, compared with saturated fatty acids, lowers plasma total and LDL cholesterol in humans (30). It has been shown that dietary linoleic acid enhances the clearance rate of LDL in humans (31) and raises receptor-mediated uptake of LDL in rabbits (32) and hamsters (33), indicating that one of the mechanisms of the hypocholesterolemic action of linoleic acid is enhanced uptake of plasma LDL by tissues. In contrast, the hypocholesterolemic action of dietary linoleic acid in rats appears to be relatively weak (34,35). With regard to this, it is worthwhile to note that in humans the activity of Δ6 desaturation is lower than that in some animal species, including rats (36). Reflecting this, the proportion of linoleic acid is higher than arachidonic acid in plasma PC of humans (37), while the proportion of arachidonic acid is higher than linoleic acid in plasma PC of rats (38). Moreover, some hypocholesterolemic substances, such as PE (39) and soybean protein (38,40), have been shown to suppress the metabolism of linoleic acid and consequently increase the proportion of linoleic acid in plasma PC or phospholipids in rats. Thus, it is likely that in rats the sup-

pression of linoleic acid metabolism is one of the most effective means to elevate the proportion of linoleic acid in plasma PC and to lower the plasma cholesterol concentration.

It is confirmed that certain lipoproteins, e.g., LDL, are taken up whole by tissues *via* receptor-mediated mechanism (41). At present, it is not known whether eritadenine enhances the activity of lipoprotein receptors. As for the hypocholesterolemic action of polyunsaturated fats (linoleic acid), it was postulated that increased fluidity of LDL receptor membranes due to incorporation of polyunsaturated fatty acid may participate in enhanced LDL apoprotein B catabolism in cynomolgus monkeys fed linoleic acid-rich fats (42). Since eritadenine increased the proportion of linoleic acid in liver microsomal phospholipids, the possibility that eritadenine also may increase the proportion of linoleic acid in lipoprotein receptor membrane phospholipids, and thereby may enhance the receptor activity, cannot be ruled out. In addition to lipoprotein receptor activity, the molecular species of plasma lipoprotein PC can affect the uptake rate of lipoprotein. Kadowaki *et al.* (43) have demonstrated that the uptake of reconstituted HDL cholesterol by perfused rat livers was largely influenced by the PC molecular species used for the reconstitution of HDL. They showed that the uptake of cholesteryl oleate of reconstituted HDL by the liver was most stimulated by 16:0-18:2 PC of five PC molecular species tested (16:0-18:2, 16:1-16:1, 18:0-18:2, 18:1-16:0, and 20:1-20:1). These results were explained in terms of different susceptibility of lipoprotein PC molecular species toward hepatic lipase, since the hydrolysis of HDL phospholipids by hepatic lipase is necessary for the subsequent uptake of HDL constituents (44). Indeed, 16:0-18:2 PC incorporated into the reconstituted HDL was most hydrolyzed by hepatic lipase *in vitro* (43). The uptake of the remnants of chylomicron (45) and VLDL (46) was also suggested to be mediated by hepatic lipase. These findings suggest that the phospholipid molecular species composition of plasma lipoproteins may participate in the regulation of plasma cholesterol concentration *via* the uptake process of plasma lipoproteins by the liver. Unlike the model experiments, it is not known whether some hypo- or hypercholesterolemic substances in the diet exert their action actually through such a mechanism. However, it seems reasonable to deduce that dietary eritadenine-induced alteration of plasma PC molecular species profile may be associated with the hypocholesterolemic action of eritadenine, since one of the most prominent changes in plasma PC molecular species caused by eritadenine is an increase in 16:0-18:2 PC.

The correlation analysis showed that most of the molecular species containing 18:1 or 18:2 in the *sn*-2 position and all of the molecular species containing 20:4, 22:5, or 22:6 have significant negative and positive correlations with plasma total cholesterol concentrations, respectively (Table 2). These results, however, must be carefully interpreted, since the correlation does not necessarily indicate the cause–effect relationship. For instance, it has been shown that dietary supplementation with 22:6n-3 increases the proportion of 22:6n-3 and 20:5n-3 in plasma PC with concomitant decrease in plasma cholesterol concentration in rats (47), indicating that plasma PC molecular species containing 22:6 or 20:5 is not necessarily associated with hypercholesterolemia. Nonetheless, plasma PC molecular species may be roughly and conveniently divided into two groups in relation to the plasma cholesterol concentration; one group consists of those molecular species containing 18:1 or 18:2 in the *sn*-2 position, and another group consists of those containing 20:4, 22:5, or 22:6. The fact that there was a significantly negative correlation between the plasma total cholesterol concentration and the proportion of plasma PC molecular species containing 18:1 or 18:2 in the *sn*-2 position (Fig. 4) appears to support that suppressed metabolism of linoleic acid (and probably 18:1) leads to reduction of plasma cholesterol, at least in part, through increases in certain plasma PC molecular species containing 18:2 and 18:1. However, the detailed mechanism remains to be further elucidated.

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Effect of Trifluoperazine on Certain Arterial Wall Lipid-Metabolizing Enzymes Inducing Atherosclerosis in Rhesus Monkeys

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ABSTRACT: The effect of trifluoperazine (TFP) was investigated on arterial wall lipid-metabolizing enzymes like acyl-CoA:cholesterol acyltransferase (ACAT) and cholesterol ester hydrolase (CEH) in rhesus monkeys. The activity was determined in aortic wall homogenates obtained from rhesus monkeys fed an atherogenic diet coupled with intramuscular injections of adrenaline and TFP. Although TFP had no significant effect on serum cholesterol and triglycerides, it decreased significantly the formation of atherosclerotic lesions by decreasing the esterification of cholesterol, by inhibiting ACAT and enhancing its utilization by activating CEH. Hence, the preventive effect of TFP on the development of atherosclerosis in rhesus monkeys is mediated through its ability to influence the activities of arterial wall lipid-metabolizing enzymes like ACAT and CEH.

Lipids 32, 867–872 (1997).

Atherosclerosis is one of the major causes of death in both developed and underdeveloped countries. This slowly progressive disease begins in childhood and does not become manifest until middle age or later (1). In both man and experimental animals, the most striking feature of this disease is its focal topography. Although classified as a systemic disorder, it does not occur randomly but rather at specific, so-called lesion-prone sites that differ structurally and functionally from nonlesion-prone sites. These lesion-prone areas are characterized by increased permeability of the endothelium to plasma proteins, such as albumin, fibrinogen, and low density lipoprotein-cholesterol. In animals challenged with a diet high in fat and cholesterol, these sites also show a marked increase in intimal cholesterol accumulation (2). Rhesus monkeys are potentially the most useful animal models to study atherosclerosis, as they are phylogenetically close to humans, develop arterial lesions similar to those seen in people, and develop changes in their lipoprotein profiles in response to a cholesterol-containing diet as observed in humans (3,4).

In experimental animals as well as humans, the metabolic activity of the arterial wall constitutes an approach for evaluating the pathogenesis of atherosclerosis (5). The arterial wall and its main structural component, the smooth muscle cell, are metabolically active tissue; hence it is anticipated that most enzymes characteristic of living matter are to be found in this tissue and that their activities are affected by changes in the microenvironment (6). Many membrane-active compounds known to inhibit transition from bilayer to micellar domains within membranes, such as chloroquine, 17-β estradiol and chlorpromazine, can suppress the formation of atheromatous lesions by changing the microenvironment of the arterial wall (7–9). In addition, a number of antiproliferative and antiinflammatory drugs, affecting the cellular, lipid and fibrous components of the lesion, prevent the formation of atherosclerotic lesions without reducing the serum lipid levels (10). Similarly, trifluoperazine (TFP), an antipsychotic drug belonging to the phenothiazine class of compounds and a potent inhibitor of calmodulin and protein kinase C, also acted as a preventive agent against cholesterol-induced experimental atherogenesis in rabbits without affecting the serum lipid profile because of its capacity to interact with cellular membrane phospholipids, leading to an alteration in membrane organization (11–13).

A literature survey showed that little work has been reported using TFP as an antiatherosclerotic agent especially in the arterial wall tissue of rhesus monkeys. In the present work, we studied the effects in atherogenic rhesus monkeys of TFP on their lipids and lipoproteins profile, and on the activities of acyl-CoA:cholesterol acyl transferase (ACAT) and cholesterol ester hydrolase (CEH), two important lipid-metabolizing enzymes of the arterial wall (14,15), which are important in the onset of atherosclerosis.

MATERIALS AND METHODS

Eighteen healthy tuberculosis-negative, adult male rhesus monkeys, 4–5 kg in weight, were acclimatized in the animal house for a period of 2 mon before starting the experiment. The animals were fed a normal diet (wheat flour) and fruits. Water was provided *ad libitum*. A course of broad-spectrum antibiotic (streptomycin; Indian Drugs and Pharmaceuticals Ltd. Rishikesh, India) and a deworming agent (Numental sus-

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; CEH, cholesterol ester hydrolase; TFP, trifluoperazine.

pension, Searle, Mumbai, India) were given to each animal during the acclimatization period.

After 2 mon, the animals were divided into three groups of six monkeys each, selected on the basis of matching body weight and basal serum lipid profiles, and daily were given the following for 8 mon: Group I (control): normal diet (200 g wheat flour containing 11.8% protein, 1.5% fat, 71.2% carbohydrates, 1.2% fiber, 1.5% minerals) + 15 g butter (containing 81% fat, 2.5% minerals). Group II: atherogenic diet (200 g wheat flour, 15 g butter, and 1 g cholesterol) along with intramuscular adrenaline (250 µg/animal) (Bengal Immunity Ltd., Calcutta, India), to enhance the production of atheromatous lesions. Group III: atherogenic diet, intramuscular adrenaline (as in Group II), and intramuscular TFP (1 mg/animal, or 10 µM in blood) (Espazine, Mumbai, India).

In all groups, the following investigations were performed at 0 d, 4 mon, and 8 mon: (i) clinical: The general condition, body weight, and blood pressure of each monkey were monitored; (ii) biochemical: The extent of induced hypercholesterolemia and hyperlipidemia was monitored by measuring serum total cholesterol and triglyceride by the methods of Zak and of Gottfried and Rosenberg, respectively (16,17). The major lipoprotein fractions, low density and high density lipoproteins, were separated by a single, discontinuous, density-gradient ultracentrifugation (Beckman Instruments, Nyon, Switzerland) in a vertical rotor according to the method of Chung *et al.* (18).

For all lipid and lipoprotein assays, blood was drawn from the femoral vein of overnight-fasted monkeys. For obtaining plasma, 0.1% EDTA (Sigma Laboratories, Wadala, Mumbai, India) was added to the blood collection tubes, and lipoprotein fractions were separated immediately by ultracentrifugation (TV-865). The animals were fully conscious when blood pressure was measured and when blood was drawn from the femoral vein.

For enzyme assays, the animals were killed by an intravenous injection of sodium pentothal. The thoraco-abdominal cavity was opened, and the aorta of each animal was removed. Homogenates of aortic tissues were prepared by the method of Stein *et al.* (19). The aorta was excised and rinsed in ice-cold KCl-Tris buffer. The aortas were carefully freed of any adventitial tissue, finely minced, and homogenized with 10 vol KCl-Tris buffer for 1 min. The homogenate was centrifuged at 1000 rpm for 2 min, the supernatant decanted, and the sediment resuspended in 5 vol of KCl-Tris. It was rehomogenized for 1 min and recentrifuged under the same conditions. The combined supernatants, termed aortic homogenate, were used as the enzyme source.

The activities of ACAT and CEH were determined at both the lesion site and the normal site adjacent to the lesion in aortas of all the animals at the termination of the experiment (i.e., 8 mon). All the estimations were carried out in cold conditions, and enzyme activities were assayed on the same day of sacrifice.

ACAT (E.C. 2.3.1.26). Arterial wall ACAT activity was assayed in aortic homogenates by determining the amount of incorporation of oleate (Sigma) into cholesterol ester in the presence of ATP and CoA. The method was adapted from Balasubramaniam *et al.* (20). The incubation mixture, having a final volume of 0.2 mL, contained 0.1 M potassium phosphate buffer, pH 7.4, 2 mM ATP, 4 mM $MgCl_2$, 0.2 mM CoA, 2 mM dithiothreitol, human albumin (1.2 mg/assay), and 20 µg cholesterol in 2 µL acetone (all solvents from Sigma). The reaction was initiated with the addition of 0.1 M oleate. The solution was incubated for 20 min at 37°C, and the reaction was stopped by adding chloroform/methanol (2:1, vol/vol). The cholesteryl esters were isolated by thin-layer chromatography by the method of Yao and Rastetter (21), using silica gel H (Sigma). The plates were developed in the following three solvent systems: Solvent I: benzene/diethyl ether/ethanol/acetic acid (60:40:1:0.05, by vol); Solvent II: hexane/diethyl ether (94:6, vol/vol); Solvent III: hexane alone.

After drying, the plates were placed in an iodine chamber. Spots of cholesterol ester were scraped off and eluted with different concentrations of chloroform/methanol. The cholesterol was then measured by the method of Zak (16).

CEH (E.C. 3.1.1.13). The modified method of Brecher *et al.* (22) was used to determine the activity of CEH in aortic tissue by measuring the amount of cholesterol ester hydrolyzed. Cholesterol ester, dissolved in 50 µL acetone, was used as a substrate and injected directly into the aortic homogenate mixture, which was then incubated for 60 min at 37°C. Then 10 mL chloroform/methanol (2:1, vol/vol) was added to terminate the reaction. The cholesterol and cholesterol esters were separated by thin-layer chromatography by the method of Yao and Rastetter (21), as described above.

Histopathological studies. At the termination of the experiment, i.e., after 8 mon of atherogenic diet administration along with intramuscular injections of adrenaline and TFP, the aortas were taken for histopathological studies. The aortas of each animal was cut at its origin, removed together with its large branches, and fixed in buffered 10% formol saline. The aorta was split open longitudinally and stained with freshly prepared Sudan IV solution for the identification of atherosclerotic lesions (23).

Two-way analysis of variance was carried out to compare all three groups, including the control. For further comparison of test groups with the control, Bonferroni *t*-test statistics were applied; in these, the population variance is estimated by the mean square error of the analysis of variance. These statistics were performed only for those parameters for which the *F*-value was found to be significant.

RESULTS AND DISCUSSION

In the present study TFP was used as an antiatherogenic agent (24). TFP was administered to the rhesus monkeys at a level of 1 mg/animal/d along with an atherogenic diet and adrenaline, which induces hypertension that is associated with an increase in vascular smooth muscle cell (SMC) mass and abnormal growth of arterial media, similar to that observed in atherosclerosis.

TABLE 1
Effect of Intramuscular Administration of Trifluoperazine (TFP) (1 mg/animal/d) on the Blood Pressure[a] of Atherogenic (hypercholesterolemic) Male Rhesus Monkeys

Group	Zero day	4 Mon	8 Mon
I (control)	Sys 110.5 ± 0.45	110.5 ± 2.0 (0.0)[b]	112.0 ± 1.85 (1.35 ± 1.15)
	Dias 80.2 ± 0.82	80.5 ± 2.5 (0.37 ± 1.66)	82.5 ± 2.6) (2.86 ± 1.71)
II (chol + adrenaline)	Sys 120.5 ± 0.5	126.6 ± 3.6) (5.06 ± 2.05)	128.2 ± 4.01* (6.39 ± 2.25)
	Dias 81.5 ± 1.50	86.66 ± 3.2 (6.33 ± 2.35)	88.1 ± 2.22* (8.09 ± 1.86)
III (chol + adrenaline + TFP)	Sys 114.66 ± 1.91	120.22 ± 2.01 (4.83 ± 1.96)	122.0 ± 1.90 (6.40 ± 1.90)
	Dias 78.33 ± 0.80	80.00 ± 2.08 (2.13 ± 1.44)	80.00 ± 2.62 (2.12 ± 1.71)

[a]mm Hg, systolic (Sys) and diastolic (Dias); chol, cholesterol.
[b]Values in parentheses represent percentage change with respect to zero day.

During the experiment, all animals maintained good health and remained active, and no significant changes were observed in the blood pressure or amount of diet and water intake (Table 1).

TFP seemed to have no significant inhibitory effect on serum lipids, as the level of total cholesterol and triglycerides decreased by only 2.7 and 2.75%, respectively, from that of Group II (animals having atherosclerotic lesions). These parameters showed a highly significant increase, by 180 and 37%, respectively, compared to that of control animals (Group I) similar to that of Group II (Table 2). The administration of TFP did not affect the plasma low density lipoprotein– and high density lipoprotein–cholesterol, as their levels remained almost similar to the atherosclerotic group (Group II) (Table 3).

During histopathological examination of Group II animals, extensive sudanophilic staining, indicating fatty streak formation, was observed (Fig. 1), and two animals of this group showed the formation of fibrous plaque (Fig. 2). In contrast, the aortas of Group III animals (TFP-treated) showed scanty sudanophilic fatty streak formation (Fig. 3). The frequency of atheromatous plaques was significantly less in Group III than Group II (Table 4). These observations clearly indicate that TFP can prevent the formation of fatty streaks leading to atherosclerosis in the aorta of rhesus monkeys.

The change in specific activities of ACAT and CEH was also studied by the *in vitro* addition of 0, 10, 25, 50, 75, and 100 μM of TFP to the aortic homogenates of normal rhesus monkeys. With increasing concentrations of TFP, a significant decrease in the specific activity of ACAT and a nominal

FIG. 1. Aorta of atherosclerotic monkey (Group II) showing atheromatous plaque formation in the descending thoracic region. The background intimal surface shows extensive sudanophilic staining, indicating fatty streak formation.

TABLE 2
Effect of Intramuscular Administration of TFP (1 mg/animal/d) on the Serum Total Cholesterol and Triglycerides (mg%) of Atherogenic (hypercholesterolemic) Male Rhesus Monkeys

Group	Parameter	Zero day	4 Mon	8 Mon
I (control)	Total chol	100.0 ± 1.82[a]	102.0 ± 2.6	100.0 ± 2.23
	Triglycerides	60.0 ± 1.36	60.5 ± 2.8	60.8 ± 2.30
II (chol + adrenaline)	Total chol	109.22 ± 1.85	294.16 ± 5.16* (188.39 ± 3.88)[b]	310.93 ± 3.73** (210.93 ± 2.78)
	Triglycerides	69.63 ± 1.30	85.00 ± 4.01* (40.41 ± 3.40)	172.0 ± 2.31* (84.21 ± 2.3)
III (chol + adrenaline + TFP)	Total chol	105.50 ± 2.82	286.0 ± 9.16* (180.39 ± 5.98)	300.0 ± 7.32** (200.0 ± 2.51)
	Triglycerides	62.99 ± 2.6	82.66 ± 3.71* (36.62 ± 3.25)	108.0 ± 2.31* (77.63 ± 2.3)

[a]Mean ± SEM for six animals.
[b]Percentage change with respect to control (Group I); *$P < 0.05$, f1 = 4.74; **$P < 0.01$, f1 = 10.05 with respect to basal values, where f1 = degrees of freedom for greater mean square. See Table 1 for abbrevations.

TABLE 3
Effect of Intramuscular Administration of TFP (1 mg/animal/d) on the Different Fractions of Plasma Lipoproteins (mg%) in Atherogenic (hypercholesterolemic) Male Rheusus Monkeys

Parameters	Zero day	4 Mon	8 Mon
LDL-Cholesterol			
I (control)	53.96 ± 1.52[a]	53.98 ± 1.65 (+0.03 ± 1.50)[b]	53.20 ± 1.75 (–1.40 ± 1.50)
II (chol + adrenaline)	54.50 ± 1.57	215.0 ± 5.01* (+294.49 ± 3.30)	260.76 ± 1.77** (+378.45 ± 1.60)
III (chol + adrenaline + TFP)	53.94 ± 1.36 (+265.77 ± 1.50)	197.30 ± 1.76* (+337.65 ± 4.60)	236.07 ± 6.51**
HDL-Cholesterol			
I (control)	44.20 ± 1.59	44.80 ± 0.70 (+1.35 ± 1.17)	44.80 ± 0.52 (+1.35 ± 1.09)
II (chol + adrenaline)	44.80 ± 1.64	44.50 ± 0.50 (–0.66 ± 1.07)	44.20 ± 3.53 (–1.33 ± 2.59)
III (chol + adrenaline + TFP)	44.52 ± 0.96	46.02 ± 2.08 (+3.36 ± 1.52)	46.54 ± 2.81 (+4.53 ± 1.82)

[a]Mean ± SEM of six observations.
[b]Values in parentheses represent percentage change with respect to zero day; *$P < 0.05$, f1 = 4.74; *$P < 0.01$, f1 = 10.05 with respect to basal values. See Tables 1 and 2 for abbreviations.

increase in specific activity of CEH were observed (Table 5).

ACAT is the primary enzyme responsible for the esterification of cholesterol in all animal cells and is involved in its absorption, very low density lipoprotein secretion, and the formation of lipid-filled macrophages and SMC (14). In the present work, *in vitro* addition of TFP inhibited ACAT activity significantly in the normal aortic tissue (Table 5), maybe by affecting the biophysical properties of cell membrane owing to changes in the membrane–lipid fluidity as reported by Bell and Hubert (9). Moreover, Mehta and Kaul (25) reported that *in vitro* addition of TFP at a concentration of 25 μM inhibited the accumulation of free and esterified cholesterol in the SMC, by inhibiting ACAT activity in hypercholesterolemic conditions.

The activities of ACAT and CEH were determined in aortic homogenates of hypercholesterolemic rhesus monkeys, both at the normal (adjacent to the lesion) site and the lesion site. The *in vivo* administration of TFP significantly decreased the specific activity of ACAT at both the normal and the lesion sites, by 70 and 64%, respectively, as compared to the atherogenic group (Group II) (Table 6). ACAT is responsible for the esterification and storage of cholesterol in the macrophage, and it was hypothesized that the inhibition of arterial wall ACAT might prevent the formation of macro-

FIG. 2. A closer view of intimal surface of aorta of Group II monkey showing the atheromatous plaque formation.

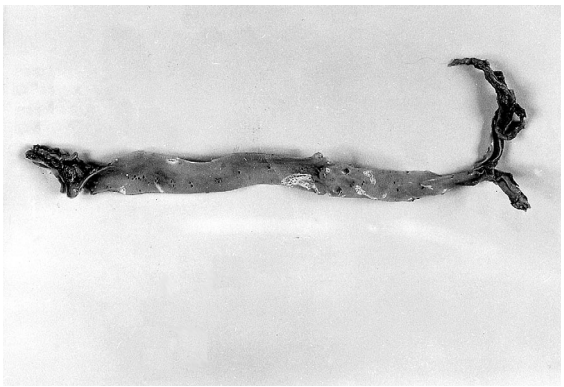

FIG. 3. Aorta of trifluoperazine-treated monkey (Group III) showing scanty sudanophilic fatty streak formation.

TABLE 4
Effect of Intramuscular Administration of TFP (1 mg/animal/d) on the Aortic Plaque Size with Frequency and Smooth Muscle Cell Number per High Power Field (HPF) of Aortic Plaques of Atherogenic (hypercholesterolemic) Male Rhesus Monkeys

Group	Aortic plaque size (μM)	Frequency of atheromatous/ fibrous plaques (%)	Smooth muscle cell number per HPF
I (control)	—	—	20 ± 10
II (chol + adrenaline)	240.69 ± 36.14	23.0	192 ± 41
III (chol + adrenaline + TFP)	45.29 ± 6.82	2.5*	77 ± 13*

*$P < 0.05$, f1 = 4.74 with respect to Group II. For other abbreviations, see Tables 1 and 2.

TABLE 6
Effect of Intramuscular Administration of TFP (1 mg/animal/d) on Specific Activity of ACAT Both at Normal and Lesion Sites in the Arterial Wall of Atherogenic (hypercholesterolemic) Male Rhesus Monkeys

	ACAT (nmol/min/mg protein)	
Group	Normal site	Lesion site
I (control)	120.0 ± 1.32[a]	
II (chol + adrenaline)	187.20 ± 21.64 (56.0 ± 11.48)[b]	312.01 ± 12.49* (160.0 ± 6.90)[c]
III (chol + adrenaline + TFP)	56.16 ± 15.33 (–53.20 ± 8.32)	112.32 ± 21.68* (–6.4 ± 11.5)

[a]Mean ± SEM.
[b]Percentage change with respect to Group I (normal site).
[c]Percentage change with respect to Group I (normal site); f1 = 4.74 with respect to normal site. *$P < 0.05$. See Tables 1, 2, and 5 for abbreviations.

phage-enriched fatty streaks and the development of clinically significant fibrous plaque (14), as observed in the present work. This antiatherosclerotic effect of TFP, achieved in the absence of cholesterol lowering, seemed to be in contrast to the reports in literature with other ACAT inhibitors such as CL 277082 (26) and cyclandelate (27), which were marked by reduced plasma cholesterol levels in rabbits along with a reduction in total aortic cholesterol content. Another ACAT inhibitor, U-73482, elevated high density lipoprotein-cholesterol in a dose-related fashion in rats (28).

In contrast to these reports, and in accordance with present observations, Bocan *et al*. (14) showed that the inhibition of ACAT within the arterial wall by specific inhibitors like CI-976 could result in the inhibition of atherosclerotic lesion progression and enhance regression without lowering cholesterol. Although TFP had no significant lowering effect on serum cholesterol and triglycerides in the present work, it did reduce the production of lesions and fatty streaks in aortas. These observations may be due to its inhibitory effect on ACAT activity.

The administration of TFP to Group III animals significantly increased the activity of CEH, by 12 and 53%, respectively, at both the normal and the lesion sites, as compared to Group II (atherosclerotic animals); whereas, compared to normal animals (Group I), the activity level was still higher, by 21%, at the lesion site (Table 7). Thus, TFP seemed to protect

TABLE 5
Effect of *in vitro* Addition of Different Concentrations of TFP on Specific Activities of Acyl-CoA:cholesterol Acyl Transferase (ACAT) and Cholesterol Ester Hydrolase (CEH) in the Arterial Wall of Male Rhesus Monkeys

TFP added (μM)	nmoles/min/mg protein	
	ACAT	CEH
0	118.32 ± 1.32[a]	19.02 ± 0.56
10	96.58 ± 0.66 (–18.37)[b]	19.51 ± 0.55 (+2.57)
25	88.73 ± 2.0 (–25.00)	19.86 ± 0.78 (+4.41)
50	67.32 ± 0.32 (–43.44)	20.22 ± 0.85 (+6.30)
75	88.73 ± 2.0 (–70.00)	19.86 ± 0.78 (+9.88)
100	29.56 ± 0.58 (–75.01)	21.68 ± 0.58 (+13.98)

[a]Mean ± SEM.
[b]Values in parentheses represent percentage change with respect to the control (0 μM TFP). For other abbreviations, see Tables 1 and 2.

TABLE 7
Effect of Intramuscular Administration of TFP (1 mg/animal/d) on Specific Activity of CEH Both at Normal and Lesion Sites in the Arterial Wall of Atherogenic (hypercholesterolemic) Male Rhesus Monkeys

	CEH (nmol/min/mg protein)	
Group	Normal site	Lesion site
I (control)	20.52 ± 0.65[a]	
II (chol + adrenaline)	19.76 ± 1.07 (–4.87 ± 0.87)[b]	16.24 ± 1.09 (–20.85+ ± 0.87)[c]
III (chol + adrenaline + TFP)	22.07 ± 0.72 (7.54 ± 0.68) (+11.69 ± 0.70)[d]	24.87 ± 0.43 (21.19 ± 0.58) (+53.14 ± 0.62)[d]

[a]Mean ± SEM.
[b]Percentage change with respect to Group I (normal site).
[c]Percentage change with respect to Group I (normal site).
[d]Percentage change with respect to Group II. See Tables 1 and 5 for abbreviations.

the aortic tissue from excess deposition of cholesterol esters by increasing the aortic wall CEH activity, thereby increasing the release of free cholesterol from the macrophages. These cells are involved in the formation of foam cells in atherosclerosis, a disease characterized by a marked increase in the content of cholesterol esters in the arterial wall owing either to a deficiency of CEH or low CEH activity (29,30). Thus, an increase in the activity of CEH seemed to be beneficial for the prevention of deposition of cholesterol esters in the atherosclerotic lesions.

The foregoing observations suggested that TFP reduced the production of fatty streaks in the aortic tissue by decreasing the esterification of cholesterol through inhibition of inhibiting ACAT and enhanced activation of CEH.

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Short-Term Effects of 3-Hydroxy-3-methylglutaryl-CoA Reductase Inhibitor on Cholesterol and Bile Acid Synthesis in Humans

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ABSTRACT: Competitive inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase improve hypercholesterolemia. However, reports about the effects of these agents on bile acid synthesis, the metabolic pathway of cholesterol, are conflicting. We studied the short-term effect of one of these agents, pravastatin, on bile acid synthesis. Six male volunteers were given 40 mg of pravastatin. Plasma mevalonate level (which reflects cholesterol synthesis) and 7α-hydroxy-4-cholesten-3-one level (which reflects bile acid synthesis) were measured every 2 h for 8 h. These plasma levels were compared to those of the same volunteers without pravastatin. Plasma mevalonate level after 2 h was lower than control (3.0 ± 1.1 ng/mL vs. 6.7 ± 2.5, mean ± SD; $P < 0.05$). This decrease continued for 8 h (2.5 ± 0.8 vs. 5.2 ± 1.5; $P < 0.05$). On the other hand, plasma 7α-hydroxy-4-cholesten-3-one level did not change until after 6 h; then at 8 h it was lower than control (15.7 ± 11.8 ng/mL vs. 24.7 ± 16.9; $P < 0.05$). According to three-way layout analysis of variance, mevalonate level was influenced by both pravastatin treatment ($P < 0.01$) and time-course ($P < 0.01$). On the other hand, the 7α-hydroxy-4-cholesten-3-one level was affected by both individual difference ($P < 0.01$) and time course ($P < 0.01$), but pravastatin treatment did not influence this compound. This indicates that bile acid synthesis was not influenced by pravastatin, although cholesterol synthesis was inhibited. The short-term inhibition of cholesterol synthesis did not affect bile acid synthesis.

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Cholesterol homeostasis in humans is maintained by two input pathways (dietary absorption and *de novo* synthesis) and two output pathways in liver (direct secretion to bile and conversion to bile acids) (1). The rate-limiting step in cholesterol synthesis is conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) into mevalonate (MVA) by HMG-CoA reductase (E.C. 1.1.1.34) (2,3). On the other hand, the first and rate-limiting step in the neutral pathway of bile acid synthesis is catalyzed by cholesterol 7α-hydroxylase (E.C. 1.14.13.7) (4,5). It is well-known that the activities of these two key enzymes change in parallel in the normal physiological state (1).

Competitive inhibitors of HMG-CoA reductase, such as lovastatin, simvastatin and pravastatin, are widely used for treatment of hypercholesterolemia (6). Many investigators have studied the effects of HMG-CoA reductase inhibitors on bile acid synthesis or bile acid secretion in different systems, including animals (7–10), patients with hyperlipidemia (11–13), biliary drainage (14), gallstone (15), and normal subjects (16,17). However, their results were conflicting: bile acid synthesis or bile acid secretion was decreased (7,14, 16,18), increased (15), or unchanged (8–13,17).

In previous studies, we reported convenient and noninvasive indices for the activity of HMG-CoA reductase (19) and cholesterol 7α-hydroxylase (20). Plasma level of MVA, the index of HMG-CoA reductase activity, has sufficient sensitivity to detect the circadian rhythm of cholesterol synthesis (21–23). The plasma level of 7α-hydroxy-4-cholesten-3-one (7α-3one), the index of cholesterol 7α-hydroxylase activity, also has enough sensitivity to detect the circadian rhythm of bile acid synthesis (20). This result indicates that they are useful for monitoring short-term changes. In this study, we report the effect of pravastatin, one of the HMG-CoA reductase inhibitors, on bile acid synthesis in humans under normal physiological conditions, using these indices.

MATERIALS AND METHODS

Chemicals. Heptadeutero-mevalonolactone (MVL-d_7) and heptadeutero-7α-3one (7α-3one-d_7) were prepared as described previously (19,20). Solid-phase column cartridges (Bond Elut SAX, Bond Elut C18, Bond Elut CN, and Bond Elut SI) were purchased from Varian (Harbor City, CA). Dimethylethylsilylimidazole (DMESI) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Solvents and other reagents were analytical reagent grade.

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Abbreviations: ANOVA, analysis of variance; DMESI, dimethylethylsilylimidazole; GC–MS, gas chromatography–mass spectrometry; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; 7α-3one, 7α-hydroxy-4-cholesten-3-one; MVA, mevalonate.

Subjects. Six healthy normal male volunteers, aged 20 to 30 yr, were studied. All subjects were free of hyperlipidemia, renal dysfunction, and hepatic impairment. Informed consent was obtained from each volunteer prior to the study, which conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Sample collection. The volunteers consumed nothing but water from 5:30 p.m. on the day before study until the end of the study in order to avoid enterohepatic circulation of bile acids. They took 40 mg of pravastatin sodium orally immediately after venous blood was collected at 9:30 a.m. Thereafter, venous blood was sampled every 2 h for 8 h. The collected blood was heparinized and immediately centrifuged at 1000 $\times g$ for 15 min, 1 mL of plasma was used at once for the determination of lipoprotein fractions, and the residual plasma was stored at −20°C until analyzed. All volunteers were used as controls (without administration of pravastatin) more than 7 d before commencement of this study.

Cholesterol and lipoproteins in blood. Plasma total and free cholesterol were measured by enzymatic method (24). Plasma low density lipoprotein and very low density lipoprotein were determined by the method of Burstein (25).

Plasma levels of pravastatin. Plasma levels of pravastatin sodium and its major metabolite, 3-hydroxy-pravastatin, were determined by gas chromatography–mass spectrometry (GC–MS) (26).

Plasma levels of MVA. The plasma level of MVA, an index of the activity of HMG-CoA reductase, was determined by the method described previously (19). In brief, 8 ng of MVL-d_7 was added to 500 μL of plasma as an internal standard. MVA in plasma was extracted and purified by serial solid-phase extractions, using Bond Elut SAX, Bond Elut C18, and Bond Elut CN cartridges. The extract was derivatized with benzylamine followed by DMESI. The resulting mevalonylbenzylamide derivative was determined by GC–MS.

Plasma levels of 7α-3one. The plasma level of 7α-3one, an index of cholesterol 7α-hydroxylase activity, also was determined according to the method described previously (20). A 7-ng aliquot of 7α-3one-d_7 was added to 200 μL of plasma as an internal standard. Then, 7α-3one was extracted from plasma by salting-out extraction and was purified by serial solid-phase extractions using Bond Elut C18 and Bond Elut SI cartridges. The extract was treated with *O*-methylhydroxylamine hydrochloride, then with DMESI. The resulting methyloxime–dimethylethylsilyl ether derivative was quantified by GC–MS.

Statistical analysis. The diurnal changes of plasma MVA and 7α-3one in controls were evaluated by Friedman's test. At each time point after administration of pravastatin, the plasma MVA, 7α-3one, and plasma lipids were compared with those levels at the same time point in controls by the paired *t*-test. The relative concentrations of MVA and 7α-3one were calculated as follows: relative concentration = level at each time/level at 0 h × 100 (%). The degree of influence of changes in relative concentration was evaluated by three-way layout analysis of variance (ANOVA). Time-course change (diurnal rhythm), pravastatin treatment, and individual difference were used as the parameters in this analysis. Probability less than 0.05 was defined as statistical significance.

RESULTS

Plasma concentration of pravastatin and its metabolite. As shown in Figure 1, levels of pravastatin sodium and its 3-hydroxy metabolite reached their maximum at 2 h after oral administration of pravastatin (69.7 ± 41.9 ng/mL and 26.7 ± 14.6 ng/mL, mean ± SD, respectively). They then decreased gradually until 8 h (6.8 ± 2.5 ng/mL and 1.1 ± 0.5 ng/mL).

Changes of plasma lipids. Table 1 shows the changes of total cholesterol, free cholesterol, low density lipoprotein and very low density lipoprotein levels with and without pravastatin. Cholesterol levels and lipoprotein levels after pravastatin administration were not found to be different from controls at the same time point.

Plasma levels of MVA. The diurnal changes of plasma MVA level with and without pravastatin are shown in Figure 2. MVA level in controls showed diurnal variation by Friedman's test ($P < 0.05$), whereas plasma MVA level at 2 h after pravastatin administration had fallen to 3.0 ± 1.1 ng/mL, significantly lower than controls (6.7 ± 2.5 ng/mL, $P < 0.05$). This decrease continued for 8 h (2.5 ± 0.8 ng/mL vs. 5.3 ± 1.5 ng/mL, $P < 0.05$).

Plasma levels of 7α-3one. Figure 3 shows the diurnal changes of 7α-3one levels with and without pravastatin. The plasma level of 7α-3one in controls did not exhibit diurnal variation, and the level after pravastatin administration was

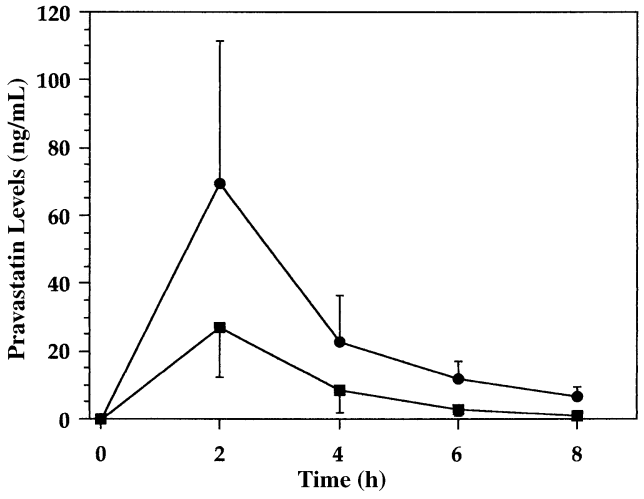

FIG. 1. Plasma levels of pravastatin sodium and its major metabolite determined by gas chromatography–mass spectrometry (GC–MS) after oral administration of 40 mg of pravastatin sodium to six volunteers. The levels of these compounds reached maximum at 2 h after administration, then decreased; ●: pravastatin sodium, ■: metabolite. Error bars express mean ± SD.

TABLE 1
Changes in Total Cholesterol, Free Cholesterol, Low Density Lipoprotein, and Very Low Density Lipoprotein After a Single Dose of Pravastatin[a]

Time	T-Cho (mg/dL)		F-Cho (mg/dL)		LDL (mg/dL)		VLDL (mg/dL)	
(h)	Control	Pravastatin	Control	Pravastatin	Control	Pravastatin	Control	Pravastatin
0	177.0 ± 26.9	182.7 ± 30.6	44.2 ± 7.3	50.6 ± 7.9	413.3 ± 130.1	400.8 ± 172.3	104.9 ± 71.1	120.4 ± 63.9
2	185.9 ± 29.5	182.9 ± 30.6	39.9 ± 13.5	48.3 ± 8.5	423.6 ± 154.3	395.7 ± 162.2	122.3 ± 68.5	107.2 ± 57.6
4	183.6 ± 37.5	179.5 ± 31.3	44.0 ± 7.4	46.9 ± 8.2	432.0 ± 153.4	386.1 ± 162.5	116.8 ± 67.2	106.2 ± 54.7
6	184.2 ± 24.6	178.4 ± 33.5	44.2 ± 6.8	47.5 ± 8.6	416.7 ± 136.6	392.0 ± 159.0	125.3 ± 77.8	111.4 ± 60.3
8	179.4 ± 27.5	180.8 ± 32.9	45.7 ± 6.4	48.9 ± 8.7	418.7 ± 151.6	395.2 ± 166.3	126.2 ± 73.3	120.6 ± 74.5

[a]Each value represents mean ± SD. T-Cho, total cholesterol; F-Cho, free cholesterol; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

not significantly different from the control until 6 h. However, the plasma level of 7α-3one at 8 h after pravastatin administration was significantly lower than controls (15.7 ± 11.8 ng/mL vs. 24.7 ± 16.9 ng/mL, $P < 0.05$).

Relative concentration of MVA and 7α-3one. The changes in relative concentration of MVA and 7α-3one are shown in Table 2. These relative concentrations of each index were evaluated by ANOVA (Table 3). Individual difference, the pravastatin treatment, and time-course change were used as the factors of this analysis. Pravastatin treatment and time course were significant factors for the change of MVA levels ($P < 0.01$). On the other hand, pravastatin treatment did not affect the levels of 7α-3one, while individual difference and time course were significant factors for the change of 7α-3one levels ($P < 0.01$).

DISCUSSION

Pravastatin and the other HMG-CoA reductase inhibitors are used widely for the treatment of hyperlipidemia. According to pharmacokinetic studies, the plasma levels of pravastatin and its metabolites reach their maximal level at 1 h after oral administration (27). In this study, the levels of pravastatin and its metabolite reached maximal level at 2 h, although we did not measure those levels at 1 h after administration. Cholesterol and plasma lipoproteins showed no significant change. These results suggest that cholesterol homeostasis does not change within 8 h after administration of pravastatin.

We measured plasma levels of MVA and 7α-3one to evaluate the single-dose effect of pravastatin on cholesterol and bile acid synthesis. We and other authors have reported that plasma levels of MVA and 7α-3one are indices of cholesterol synthesis (19,21–23,28–31) and bile acid synthesis (20,32, 33), respectively. These plasma indices can be used to monitor short-term serial change of cholesterol and bile acid synthesis.

First, we observed the diurnal variation of plasma MVA and 7α-3one levels in controls. The MVA level showed a diurnal rhythm as previously reported (21–23). On the other hand, the 7α-3one level in controls did not show diurnal change. According to our previous results, however, 7α-3one level had a circadian rhythm in healthy volunteers (20). The discrepancy between these results might be due to the entero-

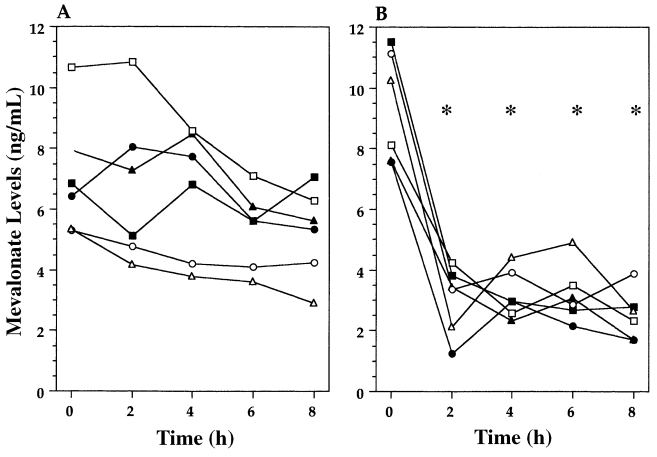

FIG. 2. Diurnal changes of plasma mevalonate (MVA) levels determined by GC–MS without (A), and with pravastatin (B). Plasma samples were collected from six healthy volunteers every 2 h for 8 h. Diurnal change was observed in controls by Friedman's test ($P < 0.05$). Plasma MVA levels after pravastatin (B) were significantly lower from 2 h to 8 h than in controls (A) by paired *t*-test ($P < 0.05$). *Significant difference ($P < 0.05$). See Figure 1 for abbreviation.

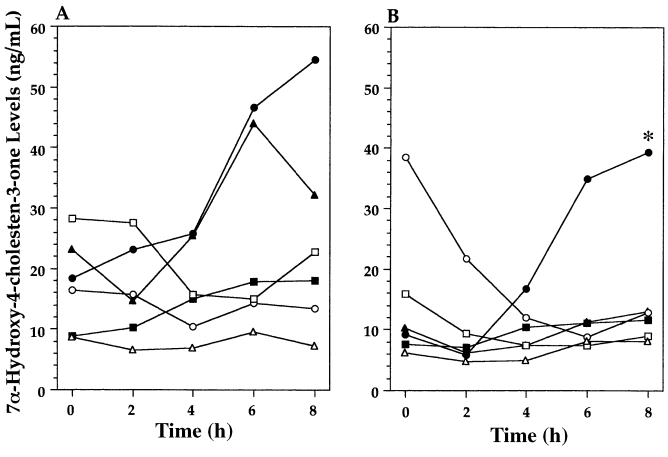

FIG. 3. Diurnal changes of plasma 7α-3one levels determined by GC–MS without (A), and with pravastatin (B). Plasma samples were collected from six healthy volunteers every 2 h for 8 h. Diurnal variation was not observed in the control. Plasma 7α-3one level at 8 h after pravastatin administration was significantly lower than in controls (A) by paired *t*-test ($P < 0.05$). *Significant difference ($P < 0.05$). See Figure 1 for abbreviation.

TABLE 2
Changes in Relative Concentration of Mevalonate and 7α-Hydroxy-4-cholesten-3-one After a Single Dose of Pravastatin[a]

Time	Mevalonate (%)		7α-Hydroxy-4-cholesten-3-one (%)	
(h)	Control	Pravastatin	Control	Pravastatin
0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
2	93.4 ± 16.8	32.9 ± 12.6	95.9 ± 21.5	68.2 ± 13.0
4	92.8 ± 17.4	34.4 ± 5.8	103.1 ± 41.0	91.5 ± 52.6
6	76.1 ± 7.4	34.9 ± 9.3	149.2 ± 70.7	139.7 ± 115.9
8	74.9 ± 16.1	26.4 ± 4.3	147.5 ± 80.1	154.9 ± 129.2

[a]Each value represents mean ± SD. Relative concentrations of mevalonate and 7α-hydroxy-4-cholesten-3-one were calculated as follows: relative concentration = levels at each time/levels at 0 h × 100 (%).

hepatic circulation of bile acid. In the previous study, volunteers took food three times in 24 h. In contrast, the volunteers in this study did not take any food except water during the study, because food intake causes the enterohepatic circulation of bile acids, which is one of the most important regulators of bile acid synthesis (34,35).

Scoppola *et al.* (31) reported plasma MVA levels after a single dose of simvastatin. The MVA level was depressed to 30–40% of baseline levels within 2 h (31). In our study, plasma MVA levels after pravastatin administration were significantly lower than control at the same time point from 2 to 8 h. We observed that MVA levels decrease to about 30% of basal level within 2 h after pravastatin. Furthermore, the change of MVA levels was evaluated by ANOVA, according to which the change of MVA level is dependent on both pravastatin treatment and time course. The result of Scoppola *et al.* (31) and our results suggest that the activity of HMG-CoA reductase was inhibited by the single dose of HMG-CoA reductase inhibitors *in vivo.* In a long-term study for administration of pravastatin, however, it has been reported that HMG-CoA reductase activity was increased (15,36). In those reports, the activities of HMG-CoA reductase were assayed after separation of the microsomal fractions from liver tissue (15,36). This inhibitor is water-soluble, and then may move into the cytosol fraction during the preparation of the microsomal fraction. Therefore, the enzyme activity of the resulting microsomal fractions probably does not reflect the *in vivo* situation.

It remains unclear whether newly synthesized or preformed cholesterol is used as substrate of bile acid synthesis dominantly. All reports on the effects of lovastatin indicate that this agent decreased bile acid synthesis or its concentration in bile (7,8,14,16,18). Vlahcevic *et al.* (7) and Björkhem (8) reported cholesterol 7α-hydroxylase activity was significantly decreased when rats were treated with lovastatin. In tracer kinetic studies (16,18), this inhibitor also reduced bile acid synthesis in healthy human volunteers. Furthermore, when patients with external bile fistula were given a single dose of lovastatin, their bile acid concentration in bile was markedly decreased (14). On the other hand, most studies on the other reductase inhibitors, such as pravastatin and simvastatin, reported that most of the bile acids are synthesized from preformed (i.e., plasma) cholesterol (9–13,17). Scheibner *et al.* (9,10) treated both rats and hamsters with pravastatin, and concluded that bile acids were synthesized mostly from preformed (i.e., plasma) cholesterol. Patients with hyperlipidemia have been treated with simvastatin (11) or pravastatin (12,13) for more than 4 wk. Neither simvastatin nor pravastatin affected bile acid concentration in bile or feces (11–13). However, Okamoto *et al.* (15) assayed both cholesterol 7α-hydroxylase activity and serum levels of 7α-hydroxycholesterol in patients with cholesterol gallstone. The serum level of 7α-hydroxycholesterol as well as 7α-3one has been shown to be an index of cholesterol 7α-hydroxylase activity (15,19). It was reported that both the enzyme activity and serum level of 7α-hydroxycholesterol were significantly increased by administration of pravastatin for more than 1 wk (15). In the present study, plasma 7α-3one level was not influenced by pravastatin treatment but influenced by individual difference and diurnal change according to ANOVA. Our results support most of the publications concerning the effects of pravastatin and simvastatin on bile acid synthesis.

In conclusion, cholesterol synthesis was inhibited more than 8 h after oral administration of pravastatin. On the other hand, a single dose of pravastatin did not affect bile acid synthesis. These results suggest that most of the bile acid was

TABLE 3
Three-way Analysis of Variance of Effects of Pravastatin on Plasma Mevalonate and 7α-Hydroxy-4-cholesten-3-one Levels[a]

	Mevalonate				7α-Hydroxy-4-cholesten-3-one			
Parameters	S	*f*	V	Fo	S	*f*	V	Fo
Individual (A)	701.3	5	140.3	1.3	139705.4	5	27941.1	32.7**
Pravastatin (B)	26132.9	1	26132.9	233.0**	1030.0	1	1030.0	1.2
Time course (C)	18155.5	4	4538.9	40.5**	45594.8	4	11398.7	13.3**
A × B	2108.9	5	421.8	3.8*	13015.4	5	2603.1	3.0*
B × C	7254.0	4	1813.5	16.2**	2106.1	4	526.5	0.6
A × C	2127.9	20	106.4	0.9	109994.5	20	5499.7	6.4**
Error	2243.3	20	112.2		17105.1	20	855.3	
Total	58723.9	59			328551.2	59		

[a]S = residual sum of squares. *f* = number of degrees of freedom. V = unbiased variance. Fo = observed value F distribution. *Significant F distribution ($P < 0.05$). **Significant F distribution ($P < 0.01$).

synthesized from preformed cholesterol in the normal physiological states.

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Oxidation of Cholesterol in Synaptosomes and Mitochondria Isolated from Rat Brains

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ABSTRACT: Cholesterol and α-tocopherol oxidations were studied in brain subcellular fractions isolated from cerebral hemispheres of 4-month-old, male Fischer 344 rats. The fractions were suspended in buffered media (pH 7.4, 37°C) and oxidized by adding (i) ferrous iron (Fe^{2+}) with or without ascorbate or (ii) peroxynitrite (an endogenous oxidant produced by the reaction of superoxide and nitric oxide). Treatment of subcellular fractions with Fe^{2+} in the presence or absence of ascorbate produced primarily 7-keto- and 7-hydroxy-cholesterols and small amounts of 5α,6α-epoxycholesterol. Since brain contains high levels of ascorbate, any release of iron could result in oxysterol formation. Peroxynitrite oxidized α-tocopherol but not cholesterol. Hence, the toxicity of peroxynitrite or nitric oxide could not be due to cytotoxic oxysterols. When synaptosomes were incubated for 5 min in the presence of 0.5 to 2 μM Fe^{2+} and ascorbate, α-tocopherol was oxidized while cholesterol remained unchanged. Thus, α-tocopherol is functioning as an antioxidant, protecting cholesterol. Diethylenetriaminepentaacetic acid blocked production of oxysterols, whereas citrate, ADP and EDTA did not. A significant percentage of mitochondrial cholesterol was oxidized by treatment with Fe^{2+} and ascorbate. Hence, mitochondrial membrane properties dependent on cholesterol could be particularly susceptible to oxidation. The oxysterols formed were retained within the membranes of synaptosomes and mitochondria. The 7-oxysterols produced are known to be inhibitors of membrane enzymes and also can modify membrane permeability. Hence, oxysterols may play an important role in brain tissue damage during oxidative stress.
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Biological membrane components such as cholesterol and phospholipids are known to undergo peroxidation which can result in structural and functional damage within the membrane. The oxidation of cholesterol gives rise to numerous products under a large variety of conditions (1). Hydroperoxy-, oxy-, and hydroxy-cholesterols are known to be formed during the oxidation of cholesterol. The majority of the oxidation products of cholesterol are known to be biologically active as cytotoxic, atherogenic, mutagenic, carcinogenic, or enzyme inhibitory substances (2). One of the goals of the current study was to obtain information on the types of oxysterols formed and their rates of formation when brain subcellular fractions were subjected to oxidative stress.

Vitamin E is the major lipid-soluble antioxidant in biological membranes. Some of our studies probed the mechanism of protection of membrane components such as cholesterol by vitamin E. If membrane vitamin E concentrations are not sufficient to provide adequate antioxidant protection, cholesterol within the membrane could be oxidized, producing various oxygenated derivatives of cholesterol. Therefore, studies relating the consumption of tocopherol with oxidation of cholesterol are important. This can be done conveniently under *in vitro* conditions by incubating brain subcellular fractions with endogenous or exogenous oxidants. Using this technique, we have previously studied the oxidation of vitamin E in subcellular fractions from brain (3). Endogenous oxidizing agents that were tested include hydrogen peroxide, lipid peroxides, or a mixture of ferrous iron (Fe^{2+}) and ascorbic acid. In addition, oxidations induced by peroxynitrite (formed from nitric oxide, a neurotransmitter, and superoxide, an oxygen radical formed during many biological reactions) are also of neurochemical interest. In this study we have investigated the oxidation of synaptosomal and mitochondrial cholesterol and α-tocopherol with Fe^{2+}, or a mixture of Fe^{2+} and ascorbic acid, or peroxynitrite. Our data can be used in selecting the appropriate oxysterol and in vitro conditions for studies of alterations of brain membrane function induced by the specific oxysterol.

EXPERIMENTAL PROCEDURES

Chemicals. All chemicals used were of reagent-grade purity from standard sources. Solvents for chromatography were high-performance liquid chromatography grade from Burdick and Jackson Laboratories, Inc. (Muskegon, MI). The hydroxy- or keto-cholesterols were obtained from Sigma Chemicals (St. Louis, MO), or Research Plus Inc. (Bayonne, NJ). Regisil with 1% trimethylchlorosilane was used for silylation of the cholesterol derivatives and was purchased from Regis

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Abbreviations: DTPA, diethylenetriaminepentaacetic acid; TMCS, trimethylchlorosilane; trivial names of sterols: cholest-5-ene-3β,7α-diol = 7α-hydroxycholesterol; cholest-5-ene-3β,7β-diol = 7β-hydroxycholesterol; 3β-hydroxycholest-5-ene-7-one = 7-ketocholesterol; 5,6,α-epoxy-5α-cholestan-3β-ol = 5α,6α-epoxycholesterol.

Technologies Inc. (Morton Grove, IL). Other special chemicals were purchased from the following sources: α-tocopherol and α-tocopherolquinone from Kodak Laboratory Chemicals (Rochester, NY) and Ficoll (Type 400) from Sigma Chemicals. Absolute ethanol was obtained from Midwest Solvents Company (Pekin, IL) and was redistilled prior to use. Ferrous sulfate heptahydrate (Sigma Chemicals) was used as the source of Fe^{2+}. Most of the reagent-grade chemicals were from Sigma Chemicals.

Biochemical assays. All experiments were repeated and confirmed with brain samples from different sets of animals on separate days. The concentrations of total proteins were determined by the Lowry technique as modified by Markwell *et al*. (4).

Chromatography of cholesterol and cholesterol oxidation products. Details of the initial treatment of the samples for analysis have already been published (5). Briefly, 2 mL ethanol containing 0.025% (wt/vol) butylated hydroxytoluene and 0.1 ml of 30% (wt/vol) ascorbic acid were pipetted into tubes containing samples for tocopherol analyses. The mixture was heated at 60°C for 30 min after the addition of 1 mL of 10% potassium hydroxide solution. Tubes were cooled, and 2 mL of water was added followed by 2 mL of hexane containing 0.025% (wt/vol) butylated hydroxytoluene. Tocopherol, cholesterol, and their oxidation products were extracted into the hexane phase by vortexing for one minute. A portion of the hexane extract was used for tocopherol assays by reverse-phase liquid chromatography with electrochemical detection (5).

The second portion of the hexane extract was used for cholesterol and oxysterol determinations (6). After evaporation of the solvent, trimethylsilyl ether derivatives of the sterols were prepared and analyzed by gas–liquid chromatography equipped with a flame-ionization detector and a capillary column under the following conditions: DB1 column, 30 m × 0.32 mm, film thickness 0.25 μm; temperature programming—oven temperature initial 50°C, initial time 3 min, program rate 30°C per min, final value 290°C, second program rate 1°C per min, final temperature 300°C and keep at 300°C for 5 min. Under these conditions, the observed retention times (in minutes) of the various sterols were as follows: cholestane (internal standard) = 14.83; cholesterol = 17.33; 7α-hydroxycholesterol = 17.02, 7β-hydroxycholesterol = 18.3, 5α,6α-epoxycholesterol = 18.75, and 7-ketocholesterol = 20.63.

Preparation of subcellular fractions from rat brain. Four-month-old, male Fischer 344 rats (Harlan Sprague Dawley, Indianapolis, IN) were used. The subcellular fractions were isolated from cerebral hemispheres by standard centrifugation methods (7). Ficoll, obtained commercially, was further purified by dialyzing a 40% (wt/vol) aqueous solution against water for 3 h. The final Ficoll concentration was estimated by using a graph relating density and concentration. Brain tissue was homogenized in 10 vol of ice-cold isolation medium containing 0.32 M sucrose, 10 mM HEPES, and 1 mM EDTA at pH 7.4, using a glass-glass homogenizer. The homogenate was centrifuged at 1300 × *g* for 3 min and the supernatant saved. The pellet was resuspended in 10 mL of the isolation medium, rehomogenized, and centrifuged at 1300 × *g* for 3 min. The pooled supernatants were centrifuged at 17,000 × *g* for 10 min to get the crude mitochondrial fraction. The resulting pellet was resuspended in 15 mL of isolation medium. Half of this suspension was layered over 11 mL of 7.5% (wt/vol) Ficoll medium which had been layered over 11 mL of 10% (wt/vol) Ficoll medium. The tubes were centrifuged in a Beckman SW 28 rotor (Fullerton, CA) at 99,000 × *g* for 45 min. The fraction at the interface between the two Ficoll solutions was removed, diluted 1:5 with isolation medium, and centrifuged for 10 min at 17,000 × *g* to isolate the synaptosomes. The purity of the fractions was tested by electron microscopy and also by estimating the activities of the marker enzymes. The activity of the Na-K-ATPase (marker for synaptosomes) was usually enriched two- to threefold in synaptosomes compared with the crude homogenate (8). Mitochondria sedimented at the bottom of the gradient and were washed once and used. The activity of the mitochondrial marker enzyme succinate dehydrogenase was enriched eight- to tenfold in the mitochondria compared with crude homogenate.

Oxidants used in the study. Most of the experiments were done using Fe^{2+} alone or in combination with ascorbate as the oxidizing agent.

Peroxynitrite was used as the oxidant in a few experiments. The compound was prepared by the method of Keith and Powell (9). Briefly, a mixture of sodium nitrite and hydrogen peroxide was acidified with hydrochloric acid, and almost immediately sodium hydroxide was added to neutralize the acid and to make the solution alkaline. The excess hydrogen peroxide was destroyed by passing the solution through manganese dioxide. The solution was then frozen. A darker yellow solution enriched in peroxynitrite separated out, was removed, and used in all experiments. The peroxynitrite concentration was estimated using an extinction coefficient of 1670 per M per cm at 302 nm (10). The peroxynitrite solution prepared was very basic. When larger volumes of peroxynitrite were added, the excess base was partially neutralized on the day of the experiment. Under these conditions, the peroxynitrite solution is stable for 2–3 h. The concentration of peroxynitrite was rechecked after partial neutralization.

Protocol for incubations. Synaptosomes were incubated in a medium (pH 7.4) simulating extracellular fluids or plasma and contained: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium phosphate, and 10 mM glucose. HEPES (10 mM) was also added for additional buffering capacity. Mitochondria were incubated in a medium containing 25 mM HEPES and 125 mM KCl. Usually subcellular fractions containing 250 μg of protein per mL of incubation media (total volume of 3 mL) were used. The oxidant concentrations and the times of incubation were varied and are given in the figure legends. After incubation of the subcellular fraction with the oxidizing agent, the tubes were placed on ice. The reaction mixture was then centrifuged for 15 min

at 17,000 rpm in a Sorvall SS 34 rotor (Sorvall Inc., Newton, CT) (35,000 × *g*) to sediment the subcellular fractions which were then analyzed for various components. When synaptosomes were incubated with a mixture of Fe^{2+} and ascorbate, more than 99% of the ascorbate was observed to have been consumed during the incubation.

Experiments with chelating agents. In these experiments, chelators were added to the buffered medium containing the synaptosomes. The following endogenous and exogenous chelators were added to all samples (final concentration = 250 μM): ADP, citrate, EDTA, and diethylenetriaminepentaacetic acid (DTPA). The samples were then treated with Fe^{+2} with or without ascorbate, incubated, and processed as described above.

Reductions with sodium borohydride. The method of Carpenter *et al*. (11) was followed. After incubation, the synaptosomal samples were saponified, extracted with hexane, and the extract was evaporated to dryness. Powdered sodium borohydride (100 mg) was added to the residue (on ice) followed by 3 mL of a mixture of methanol and diethyl ether (1:1). The solution was left overnight at room temperature in the dark. During the next morning, 2 mL of 0.5 M HCl was added, and the mixture was extracted two times with 2 mL of diethyl ether. The combined ether extracts were scrubbed with 2 mL of water, the solvent was evaporated off, and the residue was derivatized for oxysterol assays.

RESULTS

Incubation of subcellular fractions for prolonged periods of time with or without oxidants could be expected to result in damage to membranes and to release membrane components such as cholesterol into the medium. Therefore, cholesterol and oxysterols were determined in the incubation medium after the *in vitro* treatments. This was done by analyzing 1 mL of the supernatant for cholesterol and oxysterols as described in the Experimental Procedures section. We selected synaptosomes for this experiment since synaptosomes contain large amounts of cholesterol, and therefore, releases of small amounts of cholesterol into the medium would be measurable. It was found that less than 1% of total cholesterol was released. This small percentage of cholesterol found in the medium may have been due to the trapping of membranes during the removal of supernatant. Oxysterols were not detectable in the supernatant.

Since hydroperoxides may be produced during oxygen radical attack, an experiment was conducted to study the effect of reduction of oxysterols with sodium borohydride. In this experiment, synaptosomes were incubated with a mixture of 25 μM concentrations of Fe^{2+} and ascorbic acid for 3 h, and the samples were processed for oxysterols after borohydride reduction as described in the Experimental Procedures section. The results are given in Table 1. As expected, almost all (99%) of the 7-ketocholesterol was converted to the corresponding hydroxycholesterol. The oxidized samples did not show any new peaks after the borohydride treatment. Since no new hydroxycholesterols were observed, it can be concluded that hydroperoxide formation in positions other than 7 did not occur during incubations with Fe^{2+} and ascorbate. Similar results were also obtained with 50 or 100 μM concentrations of Fe^{2+} and ascorbate.

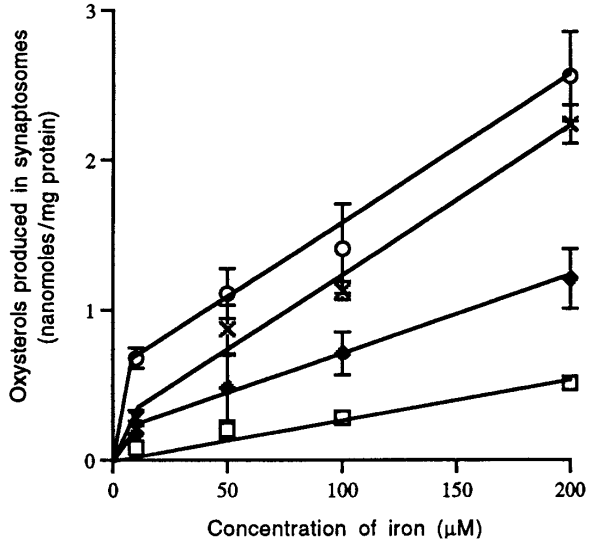
The effect of incubating synaptosomes with varying concentrations of Fe^{2+} with or without ascorbate upon the production of oxysterols was examined. As shown in Figure 1, the production of oxysterols upon incubations with Fe^{2+} increased nearly linearly with metal concentration. It is possible that ascorbate or other chelators are released into the medium and are causing oxidation in the presence of Fe^{2+} added. Therefore, accumulation of oxysterols would be expected to be directly related to Fe^{2+} concentration. This is not the case with production of oxysterols when synaptosomes are incubated with mixtures of Fe^{2+} and ascorbate. As shown in Figure 1, the formation of oxysterols tend to reach a plateau level as the concentration of the oxidants is increased beyond 100 μM.

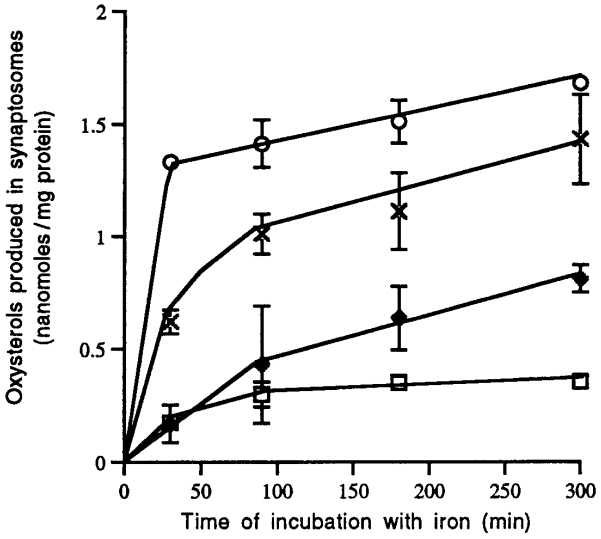
The rate of production of oxysterols when synaptosomes were incubated with Fe^{2+} with or without ascorbate is shown in Figure 2. Cholesterol oxidation does take place in the presence of Fe^{2+} alone. Since synaptosomes contain high concentrations of ascorbate, it is possible that the ascorbate is leaking out and is reacting with the added Fe^{2+} to produce the radical(s) that cause oxidation of cholesterol. Compared with treatments with Fe^{2+}, the production of oxysterols increased about fourfold when synaptosomes were incubated with both Fe^{2+} and ascorbate (Fig. 2). In both cases, there is an initial rapid phase of formation of the oxysterols which is followed

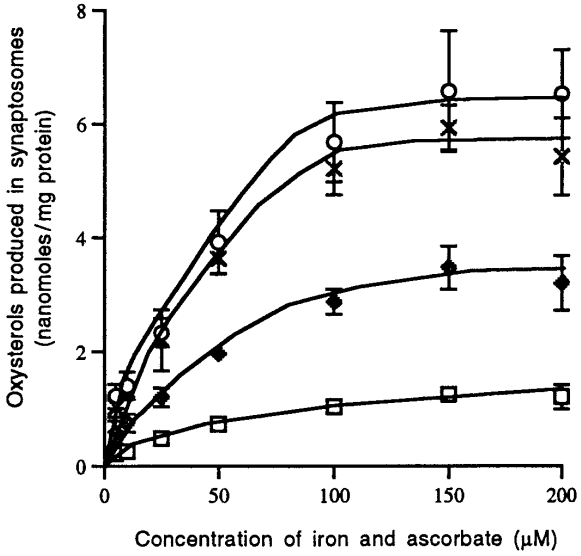
TABLE 1
The Effect of Sodium Borohydride Treatment upon the Estimated Concentrations of Oxysterols After Synaptosomes Were Incubated with a Mixture of Ferrous Iron and Ascorbate[a]

Sample type	7α-Hydroxy cholesterol	7β-Hydroxy cholesterol	5α,6α,Epoxy cholesterol	7-Ketocholesterol
Oxidized	1.82 ± 0.04*	3.36 ± 0.10	0.76 ± 0.02	3.1 ± 0.09
Oxidized and treated with borohydride	1.85 ± 0.28	6.34 ± 0.44	0.85 ± 0.08	n.d.**

[a]Samples of synaptosomes (750 μg protein in 3 mL) were oxidized by incubation at 37°C with a mixture of 25 μM ferrous salt and 25 μM ascorbate. After centrifugation, oxysterols in the pellets were determined by capillary gas chromatography. The borohydride (100 mg per tube) was added to the dried hexane extract as described in the Experimental Procedures section. *All units are nanomoles per mg protein. Mean ± SD (n = 3). **None detected.







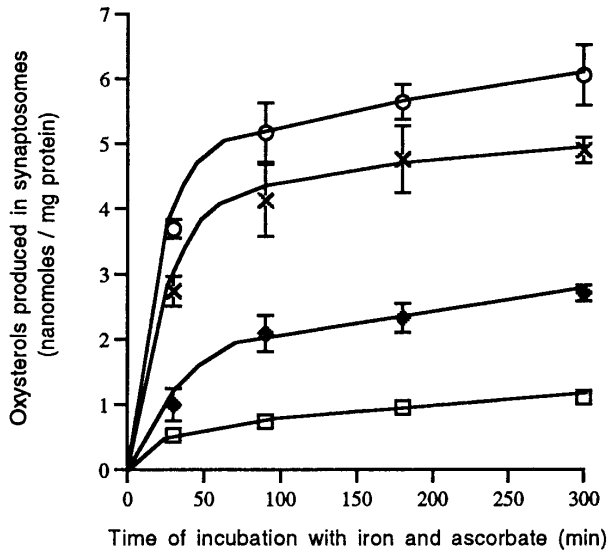


FIG. 1. The influence of concentration of Fe^{2+} or a mixture of Fe^{2+} and ascorbate upon the production of oxysterols in synaptosomes. Synaptosomes were isolated from the cerebral hemispheres of 4-month-old, male Fischer rats. They were incubated with Fe^{2+} in the presence or absence of ascorbate in a medium containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium phosphate, HEPES (10 mM) and 10 mM glucose at pH 7.4. After the incubation, the synaptosomes were separated by centrifugation and then assayed for oxysterols by gas–liquid chromatography. Cholesterol derivatives: ○, 7-keto-; X, 7β-hydroxy-; ◆, 7α-hydroxy-; □, 5α-, 6α-epoxy-.

FIG. 2. The rate of production of oxysterols from synaptosomes incubated with Fe^{2+} or a mixture of Fe^{2+} and ascorbate (100 μM). Synaptosomes were isolated from the cerebral hemispheres of 4-month-old, male Fischer rats. They were incubated with Fe^{2+} with or without ascorbate in a medium containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium phosphate, HEPES (10 mM) and 10 mM glucose at pH 7.4. After incubation, the synaptosomes were separated by centrifugation and then assayed for oxysterols by gas–liquid chromatography. Cholesterol derivatives: ○, 7-keto-; X, 7β-hydroxy-; ◆, 7α-hydroxy-; □, 5α-, 6α-epoxy-.

by a much slower phase. Hydroxyl radical production from Fe^{2+}/ascorbate mixtures is fast. It is possible that the rapid phase is due to the release of radicals by all the available Fe^{2+} and ascorbate and that the slower phase may be due to the slow leaking of ascorbate which then forms oxygen radicals in the presence of iron present on the outside.

The rate of change in cholesterol and tocopherol concentrations during the incubations of synaptosomes with Fe^{2+} alone or Fe^{2+} plus ascorbate are shown in Figure 3. Even at the first sampling time of 30 min, most of the α-tocopherol had been consumed and only a small percentage of cholesterol was oxidized. The initial phase of oxidation was examined in more detail in the next experiment. Synaptosomes were incubated for a very short time (5 min) with low concentrations of Fe^{2+} and ascorbate. The goal was to reduce the oxidative stress on the synaptosomes so that the antioxidant

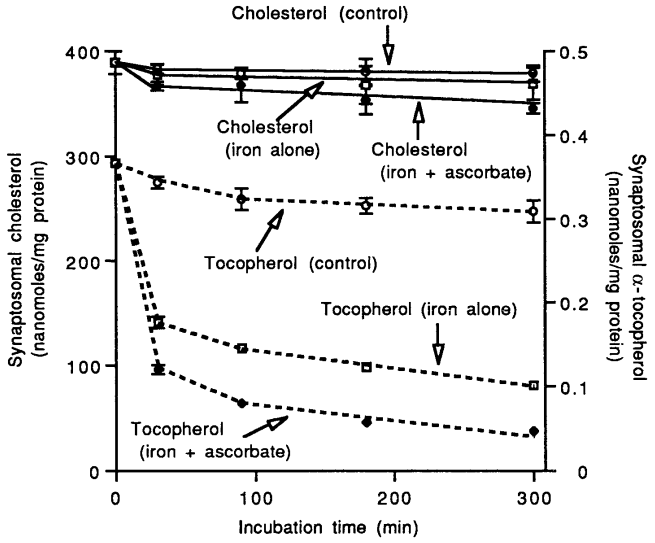


FIG. 3. The changes in concentrations of cholesterol and α-tocopherol when synaptosomes were incubated with Fe^{2+} alone or a mixture of Fe^{2+} plus ascorbate (100 μM). Synaptosomes were isolated from the cerebral hemispheres of 4-month-old, male Fischer rats. They were incubated with the oxidant in a medium containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium phosphate, HEPES (10 mM), and 10 mM glucose at pH 7.4. After incubation, the synaptosomes were separated by centrifugation and then assayed for cholesterol by gas–liquid chromatography or α-tocopherol by high-performance liquid chromatography.

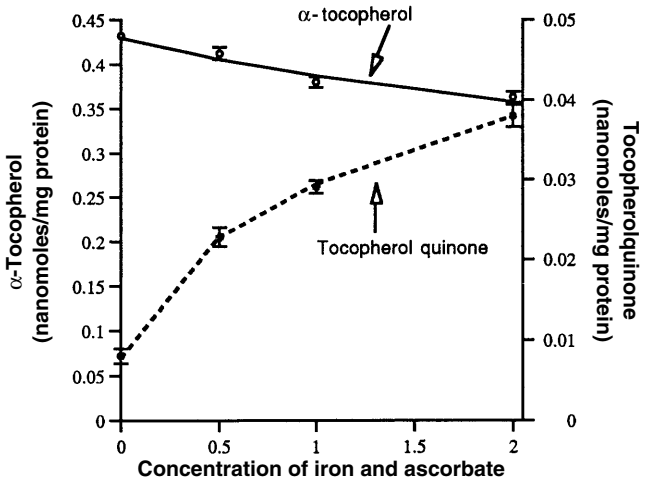


FIG. 4. Oxidation of α-tocopherol and production of tocopherolquinone when synaptosomes were incubated with low concentrations of Fe^{2+} and ascorbate for a short incubation time of 5 min. Synaptosomes were isolated from the cerebral hemispheres of 4-month-old, male Fischer rats. They were incubated with the oxidant in a medium containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium phosphate, HEPES (10 mM), and 10 mM glucose at pH 7.4. After incubation, the synaptosomes were separated by filtration and assayed for tocopherol and quinone by high-performance liquid chromatography.

activity of α-tocopherol could be demonstrated. The results are shown in Figure 4. Tocopherol was oxidized, and α-tocopherolquinone was produced at all concentrations of Fe^{2+} and ascorbate. Throughout this experiment, the concentrations of cholesterol remained unchanged within limits of experimental error, and oxysterol production was not observed. Therefore, α-tocopherol is indeed acting as an antioxidant in the membrane and is preventing the oxidation of cholesterol. It should be noted that the amount of α-tocopherol oxidized is much smaller than that of cholesterol. The efficiency of α-tocopherol as an antioxidant depends upon its ability to break free radical chains. Thus the amount of α-tocopherol needed for antioxidant protection of cholesterol would be expected to be quite small.

The influence of chelators upon the oxidation of cholesterol was studied next. Two endogenous (ADP and citrate) and two exogenous (EDTA and DTPA) compounds were used in this experiment. The amounts of 7β-hydroxycholesterol formed when synaptosomes were incubated with chelators and Fe^{+2} in the presence or absence of ascorbate are shown in Figure 5. [We chose to follow the production of 7β-hydroxycholesterol since it is one of the two main products formed and also because the sensitivity of detection of this compound is much higher than that of 7-ketocholesterol (6).] DTPA was able to completely block the production of oxysterols during oxidations induced by Fe^{+2} or Fe^{+2} and ascorbate. This may be due to the ability of DTPA to prevent the recycling of iron between the oxidized and reduced forms and to prevent the oxidative reactions driven by ascorbate and ferrous iron as observed by Burkitt and Gilbert (12).

The effect of addition of peroxynitrite to synaptosomes was tested. It was found that peroxynitrite in concentrations as high as 15 mM did not cause any declines in cholesterol or production of oxysterols. Therefore, the toxicity of peroxynitrite (and of nitric oxide, indirectly) can not be attributed to the cytotoxicity of oxysterols.

The oxidation of mitochondrial cholesterol was examined next. Mitochondria were incubated with Fe^{2+} with or without ascorbate. The changes in α-tocopherol and cholesterol concentrations during these incubations are shown in Figure 6. It can be seen that a substantial proportion of mitochondrial cholesterol is oxidized. Hence, the oxidation of mitochondrial cholesterol can be monitored reliably by measuring the decline in cholesterol concentration. This is in contrast to oxidation of synaptosomal cholesterol which cannot be followed accurately by estimating the small percentage decrease in cholesterol concentration during oxidation.

The effect of increasing the concentrations of Fe^{2+} and ascorbate upon the production of oxysterols in mitochondria was also studied. The results given in Figure 7 show that the primary products were 7-keto-, 7-hydroxy-, 5α,6α-epoxycholesterols. Thus, the oxysterols produced during oxidations of mitochondria and synaptosomes were identical in structure.

DISCUSSION

A mixture of Fe^{2+} and ascorbate was selected as oxidizing agent for three reasons. First, this mixture is known to be a

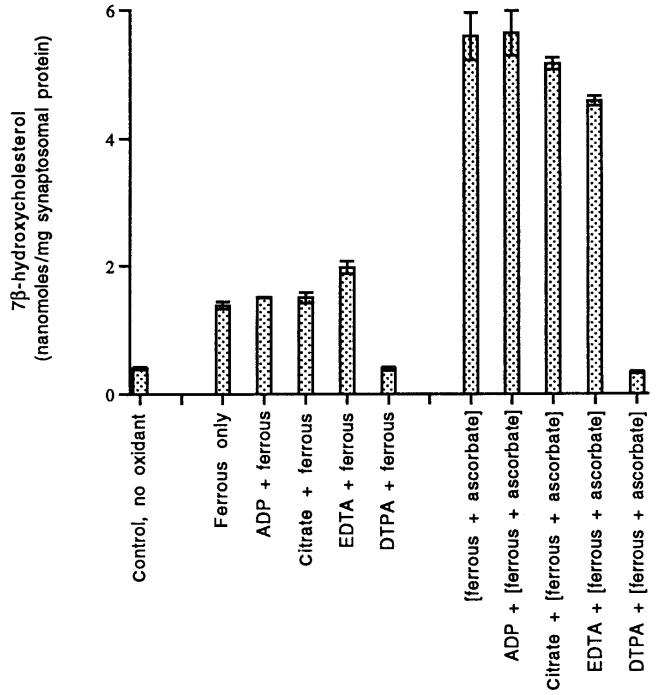


FIG. 5. The effect of addition of chelators upon the production of 7β-hydroxycholesterol when synaptosomes were oxidized with either Fe^{2+} or Fe^{2+} and ascorbate. Synaptosomes were isolated from the cerebral hemispheres of 4-month-old, male Fischer 344 rats. They were incubated in the presence of Fe^{2+} with or without ascorbate (100 μM each) in a medium containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium phosphate, HEPES (10 mM) and 10 mM glucose at pH 7.4. The chelators used were ADP, citrate, EDTA and diethylenetriaminepentaacetic acid (DTPA) at 250 μM final concentrations. After the incubation, the synaptosomes were separated by centrifugation and then assayed for oxysterols by gas–liquid chromatography.

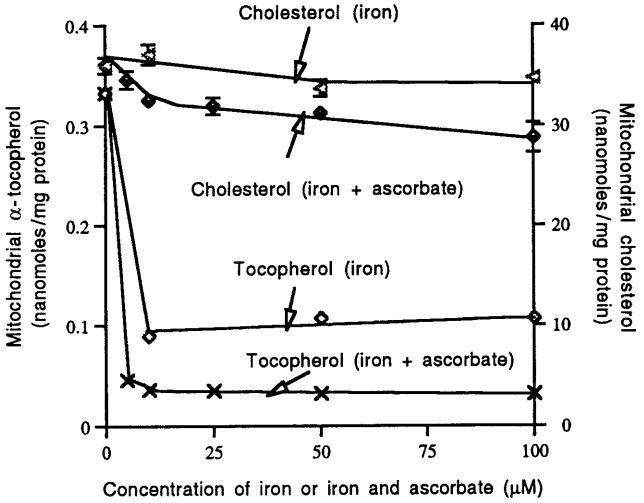


FIG. 6. Oxidation of cholesterol and α-tocopherol in mitochondria incubated with varying concentrations of a mixture of Fe^{2+} in the presence or absence of ascorbate. Mitochondria were isolated from the cerebral hemispheres of 4-month-old, male Fischer 344 rats. They were incubated for 3 h with mixtures of Fe^{2+} and ascorbate in a medium containing 25 mM HEPES and 125 mM KCl at pH 7.4. After incubation, the mitochondria were sedimented and then assayed for cholesterol by gas–liquid chromatography and for α-tocopherol by high-performance liquid chromatography.

powerful oxidizing agent. Second, the brain is characterized by high concentrations of ascorbic acid (13). High concentrations of ascorbate are present in the extracellular fluid in brain as shown by the fact that the cerebrospinal fluid concentrations of ascorbate are about two to three times that in blood plasma. Third, several areas of the brain such as the basal ganglia are known to contain high levels of iron (see review by Vatassery (14)). It also should be kept in mind that the hydroxyl radicals produced are extremely reactive and will react with the nearest membrane components from both synaptosomes and mitochondria. For example, the plasma membrane surrounding the synaptosomes will be expected to undergo oxidation, and other membranous organelles such as mitochondria within the synaptosomes may not be attacked.

The mechanism of oxidation of cholesterol and tocopherol by Fe^{2+} and ascorbate is quite complex. Hydroxyl radicals produced can abstract a hydrogen and form carbon-centered radicals that react with oxygen making peroxy derivatives. After reviewing the chemistry of oxidation of cholesterol, Lijana *et al.* (15) stated that the 7-hydroperoxide derivative is an initial product of oxidation. Gutteridge and Halliwell (16) have proposed that hydroperoxides will be formed during preparation of cell fractions and that radicals derived from the hydroperoxides will make major contributions to oxidation of membrane components. Any of these radicals could be involved in the oxidation of cholesterol and α-tocopherol.

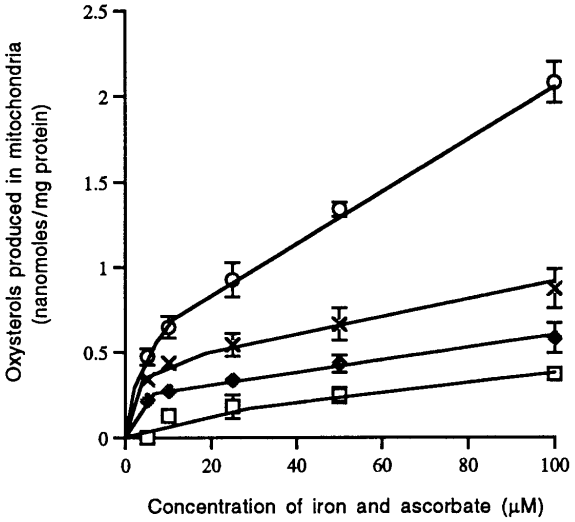


FIG. 7. Production of oxysterols when mitochondria were incubated with varying concentrations of Fe^{2+} and ascorbate. Mitochondria were isolated from the cerebral hemispheres of 4-month-old, male Fischer 344 rats. They were incubated for 3 h with mixtures of iron and ascorbate in a medium containing 25 mM HEPES and 125 mM KCl at pH 7.4. After incubation, the mitochondria were sedimented and then assayed for oxysterols by gas–liquid chromatography. Cholesterol derivatives: ○, 7-keto-; X, 7β-hydroxy-; ◆, 7α-hydroxy-; □, 5α-, 6α-epoxy-.

Few, if any, studies have examined oxysterol production in brain. However, many investigations on the oxidation of cholesterol in lipoproteins have been reported in the literature. Hydroperoxycholesterols are produced during these oxidations. These compounds are very unstable and will decompose to the hydroxy compounds even during storage of the samples in the refrigerator (personal communication: Dr. Jon Teng, Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, TX). Therefore, investigators like Carpenter *et al*. (11) extract the lipids from the samples and then treat the extract with sodium borohydride to reduce the hydroperoxides to the hydroxyl derivatives. However, sodium borohydride also reduces aldehydes and ketones to the corresponding alcohols. The results of our studies in Table 1 show that the 7-ketocholesterol was reduced to the alcohol during the borohydride treatment. Furthermore, since only 7-hydroxycholesterols were formed, the data also show that hydroperoxide substitutions in positions other than 7 did not take place during attack by hydroxyl radicals. The procedure used in this report does not include a borohydride reduction step during sample preparation. Hence, we have been able to collect data on the formation of 7-ketocholesterol.

The observation that low micromolar concentrations (about 10 μM) of Fe^{2+} in the incubation medium can cause oxidation of synaptosomal cholesterol (Fig. 1) is of special interest. As mentioned above, the concentration of ascorbate is high in the extracellular fluid of the brain. Therefore, oxygen radicals would be expected to be produced whenever Fe^{2+} is available. This suggests that the release of small amounts of iron into the extracellular environment could cause formation of cytotoxic oxysterols within membranes. Our data show that a substantial proportion of mitochondrial cholesterol undergoes oxidation (Fig. 6). Since the intracellular concentration of ascorbate is quite high in the brain, mitochondrial membrane functions dependent on cholesterol will be especially subject to change during intracellular release of iron under normal or pathological conditions. The production of oxysterols within mitochondrial membranes also could have additional deleterious effects.

Comparisons of oxidation of α-tocopherol and cholesterol in both synaptosomes (Figures 3 and 4) and mitochondria (Fig. 6) show that α-tocopherol is oxidized before cholesterol oxidation begins. In fact, when peroxynitrite is the oxidant, all of the α-tocopherol is oxidized, whereas cholesterol remains unoxidized. This confirms our previous observation that membrane cholesterol is indeed quite resistant to oxidation when compared with tocopherol (17). This is to be expected since vitamin E is involved with antioxidant protection of membrane components such as cholesterol.

Smith and colleagues (18,19), who have been pioneers in the investigation of oxidation products of cholesterol, have examined the presence of oxysterols in brain samples. They found that cholesterol derivatives with hydroxylated side chains are found in the brain. For example, they demonstrated that 24-hydroxycholesterol was synthesized from cholesterol in rat brain samples (18). Later, these authors found that this oxysterol was synthesized and metabolized in the brain (19). We did not find evidence for the formation of 24-hydroxycholesterol during treatment with Fe^{2+} or a mixture of Fe^{2+} and ascorbate. It is possible that very small quantities of this oxysterol may have been formed and our method of assay was not sensitive enough to detect this compound.

Oxidation of membrane cholesterol by mixtures of Fe^{2+} and ascorbate primarily resulted in the oxidation of the 7-position of cholesterol. The reaction products observed were mostly 7α- and 7β-hydroxycholesterols, as well as 7-ketocholesterol. We did find that smaller amounts of the 5,6-epoxide of cholesterol were formed, suggesting that the 5,6 double bond is also vulnerable to oxidation by hydroxyl radicals. These oxidation products are characteristic for autoxidation of cholesterol as pointed out by Smith (1). Sevanian and McLeod (20) have studied the oxidation of cholesterol in liposomes by hydroxyl radicals. They also found that 5,6α-epoxide, as well as 7keto- and 7β-hydroxycholesterols, were formed. Our data show that peroxidative reactions involving cholesterol within the brain are similar to those found in other tissues and *in vitro* conditions.

Some data are available in the literature on the biological properties of 7-oxysterols. The potential biological functions of the oxysterols may involve interactions with and modulation of membrane enzymes rather than cytosolic enzymes or receptors. In one study it was found that incubation of rat hepatocytes in primary culture with 7β-hydroxycholesterol resulted in the inhibition of gap junctional communication between cells (21). Theunissen *et al*. (22) found that the 7-oxy derivatives of cholesterol decreased the permeability of liposomes to glucose, and the authors attribute this to a direct effect upon membrane structural properties. Kandutsch *et al.* (23) have reported that 7-ketocholesterol is a potent inhibitor of cholesterol synthesis. Thus, the 7-oxysterols may play a role in altering membrane function and membrane enzyme activities.

Several studies have shown that some oxysterols are much more potent than cholesterol itself in inhibiting the uptake of cholesterol into fibroblasts and presumably other cells mediated by the low density lipoprotein receptor (24). Goldstein and Brown (24) suggested that the higher potency of oxysterols may be due to the fact that they are more water-soluble than cholesterol and thus will be able to diffuse out into the cytosol. We did not find that any of the oxysterols produced by the *in vitro* oxidation of the synaptosomal membranes diffused into the incubation medium. In other words, the oxysterols produced by the reaction of cholesterol with oxygen radicals remained within the membranes for a few hours during the incubations. This is a significant amount of time during which the oxysterols produced could have an effect upon membrane properties.

In summary, when synaptosomes or mitochondria from brain are treated with a hydroxyl radical-generating system, cholesterol is oxygenated primarily in the 7 position with smaller amounts of 5,6-epoxide also being formed. A sub-

stantial percentage of cholesterol in mitochondria is oxidized under these conditions. The oxysterols produced remain within the membrane for significant periods of time (as long as 5 h, the time of incubation). The formation of oxysterols during oxidative stress could be an especially important factor in altering mitochondrial function. Among the various chelators tested, only DTPA was able to block oxysterol production. The 7-oxysterols are known to alter membrane properties such as permeability and enzyme activities and may play a role in normal brain function. More importantly the possibility that since these oxysterols are known to be cytotoxic, they could cause degeneration of the brain during oxidative stress associated with pathological conditions such as Parkinson's disease and Alzheimer's disease.

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Absorption of Dietary Cholesterol Oxidation Products and Incorporation into Rat Lymph Chylomicrons

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ABSTRACT: Cholesterol oxidation products (oxysterols) induce macrophage lipid loading and accumulate in early arterial fatty streaks. The origin of lesion oxysterols has not been elucidated. The absorption of oxysterols from the diet and transport to the arterial wall by postprandial lipoprotein remnants may be a significant source. This study aimed to investigate the extent of oxysterol absorption and the effect on chylomicron composition. Cholesterol was heat-treated, causing 30% oxidation; the major oxidation products were 7β-hydroxycholesterol, 7-ketocholesterol, 5α,6α-epoxycholesterol, and 5β,6β-epoxycholesterol. Conscious lymph-cannulated rats were given a bolus gastric infusion of 50 mg oxidized cholesterol or 50 mg purified cholesterol in a vehicle of triglyceride. In the rats given the oxidized cholesterol, 6% of the oxysterol load was absorbed and incorporated into lymph chylomicrons. Rats given pure cholesterol had no increase in oxysterols above baseline levels. The incorporation of oxysterols into lymph chylomicrons differed over time with 7β-hydroxycholesterol, having peak absorption at 3 h, followed by 7-ketocholesterol at 4 h and 5α,6α-epoxycholesterol at 5 h. The absorption of oxysterols in animals given the oxidized cholesterol gastric infusate was associated with lymph chylomicron compositional changes at 2–4 h. The oxidized cholesterol-treated group had a twofold increase in the cholesterol (890 ± 84 μg vs. 440 ± 83 μg at 3 h) and triglyceride content (19.76 ± 3.4 μg vs. 8.49 ± 3.8 μg at 3 h). This led to a doubling of chylomicron size over this postprandial period, with particles having a mean diameter of 294 nm in the oxidized cholesterol-treated animals, compared to 179 nm in the purified cholesterol group. In conclusion, dietary oxysterols appear to influence postprandial lipoprotein particle size and composition. These changes may have effects on the clearance of chylomicrons from plasma, arterial delivery of oxysterols, and possible deposition in arterial lesions.

Lipids 32, 887–893 (1997).

Atherogenesis is characterized by the deposition of cholesterol and oxysterols in the wall of arterial vessels (1–3). The origin of lesion cholesterol is accepted to be derived from circulating lipoproteins, in particular low density lipoprotein (LDL). Unabated uptake by monocyte-derived macrophages of oxidatively modified LDL *via* scavenger receptors may be one source of oxysterols found in arterial lesions (4). It is thought that when delivery of lipoproteins exceeds catabolism, LDL trapped within the subendothelial space undergoes oxidative modification by cells of the arterial wall and serves as a substrate for macrophages (5,6). Alternatively, LDL may be oxidatively modified by macrophages themselves (7,8).

Cholesterol and oxysterols in arterial lesions also may be derived from chylomicrons, which are intestinally derived lipoproteins responsible for the transport of dietary lipid from the intestine to the plasma (9). Circulating chylomicrons undergo conversion to the cholesterol-rich chylomicron remnants by the action of endothelial lipoprotein lipases (9). Chylomicron remnants are delivered efficiently to the arterial wall and are selectively retained by fatty lesions (10,11). Furthermore, chylomicron remnants are avidly degraded by macrophages, and *in vitro* this leads to lipid loading (12). Feeding of cholesterol oxidation products to rabbits leads to arterial wall damage and lipid deposition (13,14), whereas purified cholesterol does not, despite persistent hypercholesterolemia. These observations, combined with several gastric gavage and venous infusion studies of oxidized cholesterol products (15–18), have led to the suggestion that atherosclerosis associated with cholesterol consumption actually reflects the level of cholesterol oxidation products in the diet rather than the amount of native cholesterol alone (19).

In man, the consumption of oxidatively modified lipids has increased, following advances in food processing, the development of convenience foods, and extended shelf-life products. However in a recent review, Smith (20) highlighted the paucity of studies investigating oxysterol absorption from the diet, metabolism, transfer among lipoproteins in blood, and assimilation into various tissues. The need for further studies and the plausible relationship between dietary oxysterols and the oxysterol content of lesions led to this study which was to investigate the absorption and incorporation of oxysterols into nascent lymph chylomicrons.

MATERIALS AND METHODS

Materials. Cholesterol (99% purity) and triolein (99%) were purchased from Sigma (St. Louis, MO). Cholesterol was de-

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Abbreviations: BSTFA, bis(trimethylsilyl)-trifluoracetamide; GC–MS, gas chromatography–mass spectrometry; LDL, low density lipoprotein; SIM, selective ion monitoring.

termined colorimetrically (Trace Scientific, catalog no: TR 13015, Melbourne, Australia). Triglycerides were measured colorimetrically (Wako, Osaka, Japan). Bis(trimethylsilyl)-trifluoracetamide (BSTFA) was purchased from Sigma. Other reagents were from Ajax (Auburn, NSW, Australia).

Preparation of gastric lipid infusions. Purified cholesterol was prepared by recrystallization of cholesterol (Sigma) from ethanol. Gas chromatography–mass spectrometry (GC–MS) analysis showed 99.9% purity. Oxidized cholesterol was prepared by heating cholesterol at 135°C for 10 h (21). GC–MS analysis of the oxidized cholesterol is shown in Table 1. The pure and heat-treated cholesterol (50 mg) were dissolved in 1 mL of chloroform then combined with 600 mg of triolein (99.9% Sigma). The solvent was evaporated under a constant stream of nitrogen in a water bath maintained at 37°C. GC–MS analysis of the purified cholesterol emulsion showed 99.9% purity.

Chylomicron isolation. Wistar rats (300 g) were anesthetized with phenobarbital (0.05 mg/kg) and the superior mesenteric lymph duct cannulated (22). In addition the duodenum was cannulated by a gastrostomy as described previously (22). Postoperatively the rats were placed in individual restraining cages with free access to water and were infused with 0.15 M NaCl through the gastric cannula at 1.5 mL/h. The animals were allowed to recover for 18 h and lymph was collected 2 h before administration of the cholesterol emulsion (50 mg purified or oxidized cholesterol) *via* the duodenal cannula. The animal model used in this study has provided a useful means to assess intestinal lipid absorption; however, the potential impact of short-term restraint on this process is presently unknown. At 30 min after giving the animals the cholesterol emulsion, infusion of saline was continued at 1.0 mL/h. Lymph was collected into tubes containing 5 mM EDTA every hour for 24 h. The lymph collected was centrifuged at 3000 rpm for 10 min to remove cells, cholesterol and triglyceride were determined, and the lymph was flushed with nitrogen and stored at 4°C for up to 48 h for GC–MS analysis. Chylomicron size and count rate were determined using an automated particle sizer (Brookhaven Industries Pty. Ltd., New York, NY).

Oxysterol analysis and quantification. Lymph oxysterols were determined using a quantitative GC–MS method in selective ion monitoring (SIM) as reported by Mori *et al*. (23). Briefly, to 500 µL of lymph in a glass tube, 2 mL of 1 M KOH/methanol and 1 µg 5α-cholestane (internal standard, 10 µg/mL in ethyl acetate) were added. The tube was flushed with N_2 and heated at 45°C for 60 min protected from light. To the saponified lymph, 4 mL of double-distilled H_2O was added, and lipids were extracted twice with diethyl ether (1 mL). The lipid extract was dried under N_2 and then derivatized with BSTFA (50 µL) and pyridine (50 µL) by heating samples at 60°C for 20 min under N_2. Samples were reconstituted after evaporation with N_2 with 1% BSTFA in heptane (50 µL) for GC–MS analysis. A Hewlett-Packard HP 5890 gas chromatograph coupled with an HP 5970 Series mass-selective detector and an HP chemstation (Palo Alto, CA), using G1034C MS chemstation software (Rockville, MD), were used. Oxysterols were resolved on an HP-1 crosslinked methyl silicone column (12 m × 0.2 mm, 0.33 mm film thickness; Hewlett-Packard), using helium as the carrier gas with an inlet pressure of 30 kPa. The mass spectrometer was operated in the electron impact mode (70 eV) and SIM was used. Four characteristic identifying ions were used for each oxysterol in SIM mode. To quantify the oxysterols calibration curves, measuring peak area vs. response of the oxysterol and the internal standard 5α-cholestane were used. Peak identification was confirmed by relative retention time and total ion mass and mass spectra comparison with external standards, as well as with the HP MS Chemstation NBS Mass Spectral Data Library of compounds to give >99% match.

Statistical analysis. The differences in lipid measurements in lymph at different time points were determined using Student's *t*-test. Area under the curve analysis also was used to determine differences in total lipid absorption. A random effect repeated measures model using PROC MIXED in SAS was used to determine relationships between oxysterol, triglyceride, and cholesterol content and particle size of chylomicrons.

TABLE 1
The Cholesterol Oxidation Products of Autoxidized Cholesterol[a]

Sterol	Weight (%) Mean ± SEM
Cholesterol	69.28 ± 4.31
Oxysterols	30.72 ± 1.54
7β-Hydroxycholesterol	6.96 ± 0.74
5α,6α-Epoxycholesterol	4.83 ± 0.52
5β,6β-Epoxycholesterol	4.83 ± 0.52
7-Ketocholesterol	8.62 ± 1.21
6β-Hydroxycholesterol	0.99 ± 0.05
Cholest-4-ene-3-one	0.43 ± 0.01
4,6-Cholestadiene-3-one	1.47 ± 0.05
Cholestane-3,6-dione	1.03 ± 0.04
Unknowns	1.56 ± 0.05
	100.00

[a]Cholesterol was autoxidized by heating at 135°C for 10 h.

RESULTS

The oxysterol profile following heating of cholesterol at 135°C for 10 h is given in Table 1. Of the total sterol mass, approximately 30% underwent oxidation. The major cholesterol oxidation products were the 7β-hydroxycholesterol, 7-ketocholesterol, 5α,6α-epoxycholesterol, and 5β,6β-epoxycholesterol with small amounts of other oxidation products. Only 1.5% of total oxysterols could not be identified.

The appearance of oxysterols in lymph chylomicrons was quantified in rats given 50 mg of oxidized cholesterol or purified cholesterol. Note that total lymph flow was not affected by the infusion of vehicle (49.05 ± 4.70 mL), oxysterols (50.06 ± 6.07 mL), or purified cholesterol (46.59 ± 5.12 mL). In the fasted state and prior to lipid infusion, rats showed

basal levels of 7β-hydroxycholesterol in the lymph. The mean preinfusion basal production of 7β-hydroxycholesterol was similar for purified and oxidized cholesterol-treated rats, that is, 937.68 ± 218.74 ng and 988.12 ± 260.41 ng, respectively (mean ± SEM). On giving triglyceride vehicle alone, there was no difference in lymph 7β-hydroxycholesterol levels (979 ± 257.70 ng) or any other detectable oxysterols, indicating that the basal level of secretion was not artifactual. Moreover, rats given purified cholesterol showed similar levels of lymph 7β-hydroxycholesterol pre- and postlipid infusion. In contrast, rats given oxidized cholesterol showed significant absorption and incorporation of oxysterols into lymph chylomicrons. The mass of cholesterol oxidation products incorporated into nascent lymph chylomicrons, expressed as a percentage of the oral load provided, was 6.27 ± 1.06%, i.e., 0.94 mg (mass in lymph)/15 mg (oral oxysterol load) percentage. The major oxysterols quantitated in lymph were 7β-hydroxycholesterol, 5α,6α-epoxycholesterol, and 7-ketocholesterol. However the 5β,6β-epoxycholesterol was not detected in the lymph although it formed a component of the heat-treated cholesterol. Of the oxysterols absorbed, there was no difference in the total mass of individual oxysterols in lymph with a mean 7β-hydroxycholesterol mass of 276.81 ± 64.07 μg, 5α,6α-epoxycholesterol 133.23 ± 19.48 μg and, 7-ketocholesterol 251.65 ± 52.72 μg. In addition, when the lymph mass of specific oxysterols was expressed as a fraction of the quantity in the infusate, there appeared to be no difference in the incorporation of oxysterols into chylomicrons. The percentage absorption of 7β-hydroxycholesterol was 7.91 ± 1.84%, 5α,6α-epoxycholesterol 5.47 ± 0.8%, and 7-ketocholesterol 5.81 ± 1.23%. The peak absorption of oxysterols appeared to differ, with 7β-hydroxycholesterol, 7-ketocholesterol, and 5α,6α-epoxycholesterol peaking at 3 h, 4 h, and 5 h, respectively (Fig. 1).

In order to compare oxysterol incorporation into lymph with that of unoxidized cholesterol, it was necessary to correct for basal or endogenous production. To achieve this, a basal mass of cholesterol in lymph (calculated as cholesterol secretion per hour before giving lipid × 12 h) was subtracted from the mass of cholesterol in lymph, following lipid delivery. On this basis, we calculated that the secretion of unoxidized cholesterol into lymph chylomicrons of rats given pure cholesterol had an efficiency of approximately 12% or threefold higher than the secretion of oxysterols in rats given autoxidized cholesterol.

Rats given cholesterol oxidation products secreted similar amounts of unoxidized cholesterol to rats given purified cholesterol, following lipid infusion, despite being given significantly less (30%) unoxidized cholesterol. The secretion of unoxidized cholesterol was 7.21 ± 1.12 mg (mean ± SEM) in rats given purified cholesterol and 6.51 ± 1.56 mg in rats given oxidized cholesterol; however, the mass of cholesterol infused in the pure-fed group was 50 mg, and in the oxidized-fed group 35 mg. Therefore, in order to achieve similar lymphatic secretion of cholesterol, the efficiency of cholesterol absorption in rats given oxidized cholesterol products would have needed to have been greater than in rats given pure cholesterol alone.

While the total mass of cholesterol secreted did not differ significantly between groups, there were substantial differences in the pattern of cholesterol secretion. The peak level of unoxidized cholesterol in rats given oxidized cholesterol appeared to be earlier than for rats given purified cholesterol (4 h and 6 h, respectively). At 1–3 h after gastric infusion, the oxidized cholesterol-treated animals had greater lymph cholesterol secretion of cholesterol compared to rats receiving pure cholesterol, and at 3 h, secretion was more than twofold different (890 ± 84 μg vs. 440 ± 83 μg cholesterol, respectively, $P = 0.0036$) (Fig. 2). In fact, the cholesterol content of

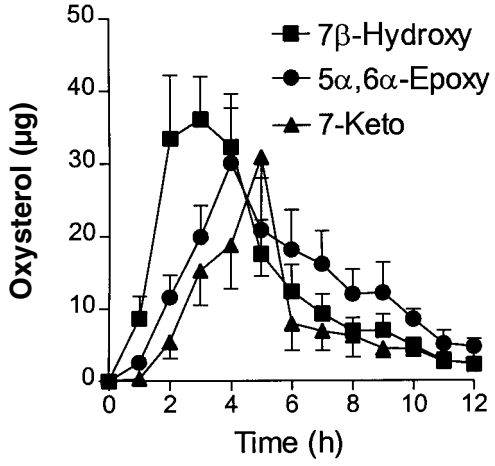

FIG. 1. The lymphatic absorption of the oxidized cholesterol products 7β-hydroxycholesterol, 5α,6α-epoxycholesterol, and 7-ketocholesterol in rats given an oxidized cholesterol emulsion *via* a gastric cannula. Values are the mean ± SEM of six rats. Lymph oxysterols were analyzed and quantitated using calibration curves with GC–MS in selective ion monitoring mode.

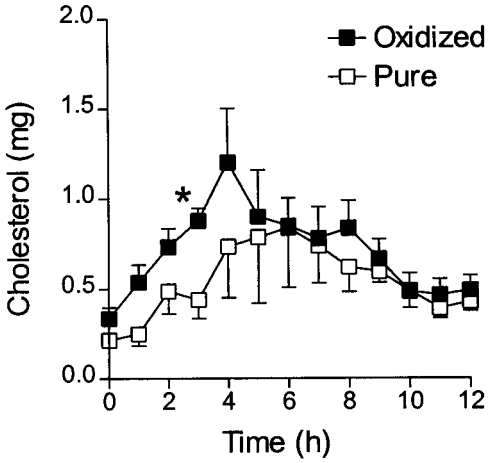

FIG. 2. The lymphatic absorption of cholesterol in lymph cannulated animals given oxidized and purified cholesterol emulsions by gastric infusion. Values are expressed as the mean ± SEM for six rats. Student's *t*-test was used at each time point; $^*P < 0.05$.

chylomicrons was found to be significantly associated with the incorporation of oxysterols ($P = 0.0008$).

The inclusion of oxysterols in dietary lipid altered the rate of lymphatic triglyceride release, although the total triglyceride secreted was not affected (oxidized cholesterol and purified cholesterol treated groups, 247.56 ± 42.76 mg and 190.98 ± 35.03 mg, respectively). Figure 3 shows that rats given oxysterols had a maximal triglyceride secretion rate of 29 mg/h at 4 h after infusion compared to 18 mg/h at 7 h after infusion for the purified cholesterol group. Overall triglyceride secretion was shown to be dependent on total oxysterol levels in the chylomicrons ($P = 0.0026$). Triglyceride secretion following absorption of the lipid load was on average twofold greater for oxysterol-treated animals at 2, 3, and 4 h compared to those given purified cholesterol. For example, the triglyceride content of the lymph in the oxidized and purified cholesterol treated groups at 3 h was 19.76 ± 3.38 mg vs. 8.49 ± 3.83 mg ($P = 0.0401$).

Triglyceride forms the bulk component of lymph chylomicrons, and hence, to a large extent, determines chylomicron particle size. The kinetics in plasma and arterial uptake of chylomicrons is regulated by size, and so the mean diameter of lymph chylomicrons was determined. Figure 4, shows that following lipid delivery, chylomicron size increased in oxysterol-treated and in native cholesterol-fed rats. However, chylomicrons isolated from oxysterol-fed rats were found to be significantly larger at 2–4 h following gastric infusion, compared to chylomicrons isolated from rats provided with pure cholesterol alone. The largest chylomicron fraction isolated from oxysterol-treated rats 3 h postprandially had a mean of 294 ± 17 nm, representing a 2.5-fold increase in size above baseline. In contrast, chylomicrons isolated from rats given pure cholesterol only attained a mean diameter of 179 ± 17 nm ($P = 0.0008$). Although triglyceride secretion increased in rats given oxidized cholesterol, the greater particle size at 2–4 h postlipid infusion was also dependent on the increased level of oxysterols ($P = 0.0001$).

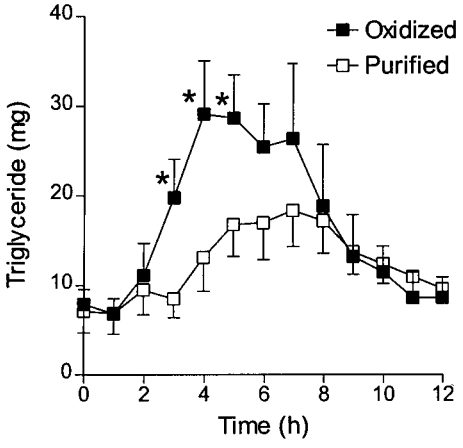

FIG. 3. The absorption of triglyceride in the lymph of animals given oxidized and purified emulsions. The lymphatic duct was cannulated in rats, and cholesterol emulsions were administered *via* a gastric cannula. Values are the mean ± SEM for six rats. Student's *t*-test was used at each time point, $*P < 0.05$.

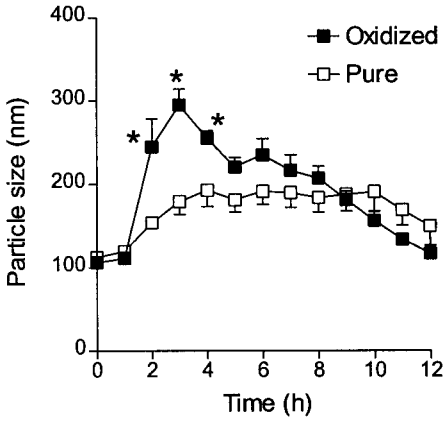

FIG. 4. The particle size of lymph chylomicrons in the oxidized and purified cholesterol-treated animals. Particle size of the lymph chylomicrons was determined using an automated particle sizer after collection of lymph every hour. Values are the mean ± SEM of six rats, $*P < 0.05$.

DISCUSSION

Oxysterols have been implicated in the onset and progression of atherosclerosis, based on observations of their proatherogenicity (20,24,25). Indeed, oxidized cholesterol products have been shown to have greater atherogenicity than native cholesterol, as evidenced by arterial damage and development of arterial lesions (15–19). The accumulation of oxysterols in atherosclerotic lesions suggests they are involved in the disease process (1–3). Oxidatively modified LDL, which is rich in oxysterols (23), has been considered the primary cause of endothelial damage (26,27) and a source of arterial macrophage cholesterol loading, which leads to the development of arterial fatty streaks (8,29,30). Alternatively, dietary oxysterols, found primarily in cholesterol-rich processed foods, might be preferentially transported to the arterial wall by postprandial lipoproteins and also may be a source of lesion oxysterols. This is plausible, given that postprandial chylomicron remnants are avidly taken up and retained by the arterial wall *in vivo* (10,11) and induce lipid loading in macrophages *in vitro* (12). However, there is a paucity of data with respect to oxysterol absorption from the intestine, secretion into circulation, and assimilation in tissues. The aims of this study were both to determine the extent of dietary oxysterol incorporation into lymphatic chylomicrons, and to compare the efficiency of lymphatic secretion with native cholesterol.

Autoxidized cholesterol products were generated following heating of pure cholesterol at 135°C for 10 h. The oxysterols produced were found to be qualitatively similar to those found in common cholesterol-rich food products (31) and mixed diets (32), with significant amounts of 7β-hydroxycholesterol, 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol, and 7-ketocholesterol. Approximately 30% of the cholesterol was oxidized following heating, and we were able

to identify 98% of the oxidation products. The oxysterol content of cholesterol-rich processed foods such as egg, milk and cheese powders, and other processed foods such as biscuits in the ppm is well established (20,31). The extensive use of egg and milk powders in a myriad of processed products suggests that ingestion of oxysterols from the diet is significant. Indeed Emanuel *et al*. (33) reported an increase in postprandial plasma levels of oxysterols after giving patients dried egg powder containing ~230 ppm oxysterols. Cholesterol oxidation products may form a small but potentially significant amount of total lipid intake.

In rats, we found substantial incorporation of dietary oxysterols in lymph chylomicrons. However, there were significant differences in the type of oxysterols absorbed, the patterns of lymphatic release, lymphatic secretion of other dietary lipids, chylomicron size, and chylomicron composition. Secretion of oxysterols into lymph would be dependent on the sum of absorption, processing, and packaging within the enterocyte. This study did not enable us to distinguish among these three processes, but rather provides us with an overview of the consequence of dietary oxysterol delivery.

Oxysterols have the potential to bind to "carrier" proteins such as albumin in addition to lipoproteins. However, following isolation of the lymph chylomicron fraction by ultracentrifugation, we found no detectable oxysterols in the infranatant. It would seem unlikely, therefore, that dietary oxysterols were delivered *via* the portal system in the absence of lipoprotein transport. Consistent with this, several groups have shown that dietary oxysterols were found exclusively associated with the triglyceride-rich lipoprotein fraction in plasma (33,34). It is likely that oxysterols not associated with lymph chylomicrons were excreted, although this was not specifically determined in this study.

Of the four major cholesterol oxidation products infused, only three appeared in lymph. We were unable to detect 5β,6β-epoxycholesterol in chylomicrons. The selective appearance or exclusion of oxysterols suggests either absorbance across intestinal epithelial cells differed with isomeric forms of cholesterol oxidation products or, alternatively, differential processing occurs within the enterocyte. Bile acids are necessary for efficient absorption of hydrophobic sterols, presumably including oxysterols. It is unlikely that 5β,6β-epoxycholesterol fails to form micelles with bile; hence, we would suggest that 5β,6β-epoxycholesterol either is degraded in intestinal epithelial cells, or simply not incorporated into chylomicrons.

The appearance of oxysterols in lymph chylomicrons differed quantitatively and qualitatively from native cholesterol. Expressed as a percentage of the dose administered, the incorporation of oxysterols into lymph chylomicrons was less than for native cholesterol, even after allowing for basal cholesterol production. There are several possible explanations for these observations. Absorption of lipids would be a finite process; so perhaps uptake was saturated under our infusate regimen. Consistent with this suggestion is the observation that, despite a smaller dose of native cholesterol in the oxysterol-fed rats, secretion of native cholesterol in oxysterol-fed rats was the same as in those given cholesterol alone. Alternatively, absorption of oxysterols may simply be less efficient, representing competition for, or a decrease in, the rates of esterification, as the primary form of cholesterol secreted in chylomicrons is esterified (35). However, it has been reported that the majority of oxysterols in lymph chylomicrons are nonesterified (36). From an alternative perspective, if absorption of oxysterols was similar or indeed greater than native cholesterol, lower rates of secretion would be a consequence of decreased incorporation into chylomicrons. Although this seems unlikely, the absence of 5β,6β-epoxycholesterol from lymph chylomicrons would be consistent with this possibility.

In a similar study Osada and colleagues (35) investigated oxysterol absorption in lymph-cannulated rats. There were several observations in that study which differed from the present report. Osada *et al.* found that the appearance of oxysterols in lymph was linear 24 h after administration of the diet, whereas in this study incorporation of oxysterol in lymph chylomicrons peaked 3–5 h after lipid delivery and returned to baseline levels at approximately 12 h. In addition, Osada and co-workers found that approximately 30% of the oxysterol load appeared in lymph chylomicrons which is significantly greater than the 6% absorption of oxysterols observed in this report. The differences between the two studies might be explained in the nature of the gastric infusates. Firstly, Osada *et al*. delivered a greater mass of oxysterols relative to pure cholesterol (88% vs. 30% of total sterol load, respectively). The lipid emulsion was also dissolved in sodium taurocholate, a bile acid salt, which may have aided emulsification and absorption. Consistent with this possibility is that, in the absence of exogenous emulsifying agents, the postprandial lipemic response following a lipid load in rats and in man normally displays "bell-shaped" kinetics (36). Nevertheless, both reports demonstrate that oxysterols can be delivered into circulation associated with chylomicrons.

The presence of oxysterols in the infusate enhanced the chylomicron triglyceride secretion rate into lymph 2–4 h postpresentation of the lipid load. This is a surprising observation which may have reflected increased absorption of triolein and/or increased esterification of monoglyceride within the enterocyte. A greater rate of triglyceride release was not compensatory to decreased cholesterol incorporation, which was also enhanced in oxysterol-treated rats. Furthermore, the increase in chylomicron triglyceride and cholesterol content in oxidized cholesterol-fed rats was not a consequence of greater chylomicron biosynthesis because particle number remained constant. Rather, chylomicrons isolated from oxysterol-fed rats were substantially larger than those isolated from rats given pure cholesterol alone. The clearance from plasma of larger chylomicrons is quite different from smaller chylomicrons (37); however, the impact of oxysterols on this process remains to be investigated.

When clearance of chylomicrons is delayed such as in type III hyperlipoproteinemia (38), familial hypercholesterolemia (39), or in secondary disease states such as diabetes (40),

there is an increased frequency of atherogenesis. A reduced clearance from plasma is thought to result in increased arterial delivery and retention of postprandial lipoproteins (11). Oxysterols may affect the metabolism of chylomicrons in plasma, including interaction with lipoprotein lipases and uptake of chylomicron remnants by high-affinity mechansims. Redgrave *et al*. (41) have shown that the apolipoprotein complement of chylomicrons is significantly altered by the cholesterol content on the surface of the lipoprotein molecule. Similarly chylomicrons containing oxysterols may alter the apolipoprotein complement, in turn affecting their metabolism. Mortimer *et al*. (42) showed 7β-hydroxycholesterol incorporation into chylomicrons impaired the clearance of cholesterol ester, an indicator of remnant uptake. Oxysterols incubated with macrophages also stimulate sterol accumulation in macrophages (43). Oxysterols also might exacerbate the atherogenicity of postprandial remnants. Chylomicron remnants serve as excellent substrates for arterial macrophages and can induce foam cell formation (10–12). Indeed, lipid-laden macrophages isolated from early human atheroma contain mainly 7β-hydroxycholesterol (1), and advanced lesions contain a number of oxysterols which we have shown to be secreted into lymph associated with chylomicrons, including 7β-hydroxycholesterol, 7-ketocholesterol, and 5α,6α-epoxycholesterol (1–3). It is plausible that the inclusion of oxysterols into lymph chylomicrons delays their clearance from plasma with the consequence of increasing exposure and delivery of lipid to the arterial wall. We can conclude from this study that oxysterols in atherosclerotic tissue might, in part, be derived from dietary sources.

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Refeeding Varying Fatty Acid and Cholesterol Diets Alters Phospholipids in Rat Intestinal Brush Border Membrane

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ABSTRACT: Refeeding a diet initially given shortly after weaning results in a different adaptive change in the *in vitro* intestinal uptake of sugars and lipids than if the diet is given for the first time at a later age. This study was undertaken in rats to test the hypothesis that changes in nutrient uptake associated with refeeding diets containing beef tallow (S), beef tallow plus 1% cholesterol (Sc), fish oil (F), or fish oil plus cholesterol (Fc) are associated with changes in the brush border membrane (BBM) phospholipids and phospholipid fatty acids. Weanling Sprague-Dawley rats were fed *ad libitum* one of the four diets. At 35 d of age (about 2 wk after weaning), the rats were maintained on either the same diet used at weaning, or were switched to one of the other semisynthetic diets which were then fed for a further 7 wk. At week nine (2 + 7) the rats were either continued on the same diet or were switched back to the original diet for 2 wk (2 + 7 + 2). The groups of animals which were compared included SSSc vs. ScSSc; ScScS vs. SScS; FFFc vs. FcFFc; and FcFcF vs. FFcF. Refeeding S, Sc, F, or Fc had no effect on food consumption or on body weight gain. Refeeding Fc resulted in increased ileal BBM total phospholipids, whereas rechallenge with F resulted in a decline in the jejunal BBM ratio of phospholipid/cholesterol. Refeeding Sc resulted in a decrease in the ileal BBM phosphatidylcholine (PC). In rats rechallenged with Fc, there was increased ileal BBM sphingomyelin (SM), increased ileal BBM phosphatidylethanolamine (PE), decreased ileal BBM PC/PE, and an increased ileal BBM SM/PC. Refeeding had no effect on the fatty acyl constituents of the jejunal or ileal BBM PC or PE. These results suggest that there are late effects of the early introduction of dietary cholesterol on intestinal BBM phospholipid content and composition that may contribute to the previously reported changes in intestinal nutrient absorption.
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Nutrient absorption by the intestine is subject to adaptation (1). For example, there is increased absorption of nutrients in animals with streptozotocin diabetes, or in the remaining intestine following intestinal resection; in contrast there is reduced absorption of nutrients following abdominal irradiation, with aging, or with the chronic feeding of ethanol (2,3). Alterations in intestinal absorption are associated with changes in the amount and/or type of brush border membrane (BBM) phospholipids of the BBM (4–9). These BBM lipid changes may be associated with alterations in enterocyte microsomal phosphatidylethanolamine (PE) methyltransferase activity, or with changes in BBM or enterocyte microsomal activity of PE methyltransferase (10). Alterations in dietary fat unsaturation alter the intestinal transport of nutrient as well as the fatty acid composition of the BBM phospholipids (11,12).

Refeeding a diet initially given to a rat shortly after weaning results in different adaptive changes in the *in vitro* intestinal uptake of glucose, fatty acids, and cholesterol than if the diet is given for the first time at a later age (13). These transport changes are not explained by differences in the animals' food intake, body weight gain, or intestinal morphology. The signal for critical period programming may be the ratio of dietary carbohydrate to protein (14), polyunsaturated to saturated fatty acids (15,16), or long-chain polyunsaturated fatty acids to saturated fatty acids (17,18). Adding cholesterol to a low-fat diet alters the late effects of early nutrition (13,19). The addition of cholesterol to a high-fat diet also influences the adaptive response of the intestine when fed early or later in life (20).

Our studies using a diet feeding program involving diets enriched with saturated fatty acids or n-3 rich oils, with or without cholesterol supplementation, have shown that such treatments are associated with alterations in the intestinal transport of sugars and lipids (20). For example, refeeding a diet supplemented with cholesterol is associated with an increase in galactose, fatty acid, and cholesterol uptake as compared with feeding a cholesterol-supplemented diet for the first time later in life. Accordingly, the present study was undertaken in rats to test the hypothesis that previously reported alterations in the intestinal transport of sugars and lipids associated with refeeding diets containing saturated beef tallow (S), beef tallow plus 1% cholesterol (Sc), polyunsaturated fish oil (F), or fish oil plus 1% cholesterol (Fc) are associated with changes in BBM phospholipids and phospholipid fatty acids.

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Abbreviations: AP, alkaline phosphatase; BBM, brush border membrane; F, fish oil diet; Fc, fish oil diet with added cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; S, beef tallow diet; Sc, beef tallow diet with added cholesterol.

METHODS AND MATERIALS

Diet sequences. The guiding principles for the care and use of laboratory animals, approved by the Canadian Federation of Biological Societies and by the Council of the American Physiological Society, were carefully observed in the conduct of this study. Weanling female Sprague-Dawley rats were purchased commercially and housed with *ad libitum* access to water and food. There were 8–10 animals in each group. Pairs of rats were housed at a temperature of 21°C, with 12 h of light and 12 h of darkness. Weanling animals were fed isocaloric semisynthetic diets containing either saturated beef tallow or polyunsaturated fish oil, with or without added cholesterol (1.0%). The four diet groups are abbreviated as: S, Sc, F, or Fc. At 35 d of age (2 wk after weaning), the rats were maintained on either the same diet used at weaning, or were switched to one of the other diets which were then fed for a further 7 wk (Table 1). At week nine (2 + 7), the rats were continued either on the same diet, or were switched back to the original diet for 2 wk (2 + 7 + 2). Eight groups of animals were compared as follows: SSSc vs. ScSSc; ScScS vs. SScS; FFFc vs. FcFFc; and FcFcF vs. FFcF.

Composition of the diets. The protein content of the semisynthetic diets was 27%, and the carbohydrate content was 40.7% (starch 20% and glucose 20.7%). The diets contained 20% (w/w) fat of either a high or a low polyunsaturated-to-saturated fatty acid ratio. The proportions of polyunsaturated, monounsaturated, and saturated fatty acids are shown (Table 2). Cholesterol was added to either the S or to the F diet to give a concentration of 1%. These diets were nutritionally adequate, providing for all known essential nutrient requirements [ingredient and concentration (g/kg diet), respectively]: fat, 200; starch, 200; casein, 270; glucose, 207.65; nonnutritive fiber, 50; vitamin mix, 10; mineral mix, 50.85; L-methionine, 2.5; choline, 2.75; inositol, 6.25, where A.O.A.C. vitamin mix (Teklad Test Diets, Madison, WI) provided the following per kilogram of complete diet: 20,000 IU of vitamin A; 2,000 IU of vitamin D; 100 mg of vitamin E; 5 mg of menadione; 5 mg of thiamine-HCl; 8 mg of riboflavin; 40 mg of pyridoxine-HCl; 40 mg of niacin; 40 mg of pantothenic acid; 2,000 mg of choline, 100 mg of myoinositol; 100 mg of *p*-aminobenzoic acid; 0.4 mg of biotin; 2 mg of folic acid, and 30 mg of vitamin B_{12}; and Bernhart Tomarelli mineral mix (General Biochemicals, Chagrin Falls, OH) was modified to provide 77.5 mg of Mn^{2+} and 0.06 mg Se^{2+} per kilogram of complete diet. The diets containing the added 1% cholesterol were designated as Sc or Fc.

Food consumption was determined twice weekly. The animals were weighed at the beginning of the study, and weekly thereafter. At study termination, the animals were anesthetized by the injection of sodium pentobarbital (800 mg/kg body weight). Intestinal weights were determined when the BBM were prepared for lipid analysis, as described below.

BBM preparation and analysis. Jejunal and ileal BBM were prepared from mucosal scrapings by homogenization, $CaC1_2$ precipitation, differential centrifugation, and density gradient centrifugation. The details of this methodology have

TABLE 1
The Feeding Regimens Illustrating the Diet Changes in Eight Groups of Animals, and Their Ages at Which the Switches Were Made[a]

	Animal age			
Diet	3 Wk	5 Wk	12 Wk	14 Wk
SSSc	S	S	Sc	Sacrifice
ScSSc	Sc	S	Sc	Sacrifice
ScScS	Sc	Sc	S	Sacrifice
SScS	S	Sc	S	Sacrifice
FFFc	F	F	Fc	Sacrifice
FcFFc	Fc	F	Fc	Sacrifice
FcFcF	Fc	Fc	F	Sacrifice
FFcF	F	Fc	F	Sacrifice

[a]S, diet containing beef tallow; F, diet containing fish oil. When cholesterol was added, these were known as Sc and Fc, respectively.

TABLE 2
Diet Fatty Acid Composition[a]

Fatty acid (% of total fatty acids)	Beef tallow	Fish oil
14:0	3.3 ± 0.04	4.6 ± 0.08
16:0	27.0 ± 0.17	18.2 ± 0.39
16:1n-7	n.d.	4.7 ± 0.19
17:0	2.1 ± 0.01	0.6 ± 0.14
18:0	58.8 ± 0.24	28.6 ± 1.37
18:1n-9	1.9 ± 0.05	6.4 ± 0.24
18:1n-7	n.d.	1.8 ± 0.06
18:2n-6	6.9 ± 0.22	9.4 ± 0.38
18:3n-3	n.d.	0.7 ± 0.06
18:4n-3	n.d.	2.6 ± 0.10
20:4n-6	n.d.	0.7 ± 0.10
20:5n-3	n.d.	14.5 ± 0.56
22:5n-3	n.d.	1.4 ± 0.05
22:6n-3	n.d.	5.9 ± 0.21
Total saturates (S)	91.1 ± 0.27	52.0 ± 1.82
Total monounsaturates (M)	1.9 ± 0.05	12.9 ± 0.48
Total polyunsaturates (P)	6.9 ± 0.22	35.1 ± 1.35
Total n-9	1.9 ± 0.05	6.4 ± 0.24
Total n-7	n.d.	6.5 ± 0.24
Total n-6	6.9 ± 0.22	10.1 ± 0.42
Total n-3	n.d.	25.0 ± 0.94
M/S	0.02 ± 0.00	0.26 ± 0.02
P/S	0.08 ± 0.00	0.72 ± 0.06
n-6/n-3	n.d.	0.40 ± 0.00
Unsaturation index	16 ± 0	161 ± 6
Diet preparation (per kg diet)		
Basal diet	800 g	800 g
Hydrogenated beef tallow	180 g	80 g
Safflower oil	20 g	20 g
Fish oil	—	100 g

[a]For diets containing cholesterol, 1% cholesterol was added. No 22:1 was present in the diet; n.d., not detected.

been published (4,21). Protein was determined according to the method of Lowry *et al*. (22). The BBM markers that were assayed included sucrase (23) and alkaline phosphatase (24). In addition, Na^+K^+-ATPase (25), NADPH cytochrome C reductase (26), β-glucuronidase (27), and DNA (28,29) were assayed as markers of basolateral membrane, microsomes, lysosomal membranes, and nuclear material, respectively. The activities of these various enzymes are linear with respect to the amount of membrane protein added to the assay system. All assays of enzyme activities were performed at substrate concentrations that yield maximal activity rate values.

Lipid extractions of the isolated BBM were carried out immediately after preparation, according to a modification of the methods of Bowyer and King (30) and Folch *et al*. (31). Free cholesterol and cholesterol esters were determined enzymatically from lipid extracts (32). The total phospholipid content was assayed from lipid extracts (33), and the phospholipid composition was assayed by thin-layer chromatography (34).

Lipid extraction and analysis of phospholipid fatty acid composition. The final BBM preparation was suspended in the supernatant, and aliquots were taken for immediate lipid extraction according to a modification (30) of the method of Folch *et al*. (31). Ethoxyquin was used as an antioxidant. Lipid extracts were dried under nitrogen at 50°C, reconstituted in chloroform, and spotted onto silica gel H thin-layer chromatography plates (20 × 20 cm, 250 μ thickness; Analtech, Newark, DE). The plates were developed in chloroform/methanol/2-propanol/triethylamine/deionized water (60:18:50:26:14, by vol) to separate the individual phospholipids. The phospholipid spots were visualized with 0.03% (wt/vol) 2′,7′-dichlorofluorescein in 0.01 M NaOH under ultraviolet light. Phosphatidylcholine (PC) and PE spots were scraped into tubes for transesterification of the fatty acid components using 14% boron trifluoride in methanol (Sigma, St. Louis, MO), as described previously (35). A known amount of nonadecane was used as the internal standard. Phospholipids were refluxed with boron trifluoride in methanol/hexane (1.5:2.0, vol/vol) for 50 min at 90–100°C. Deionized water (1.0 mL) was added to the tubes, which were then allowed to stand 90 min before being centrifuged at 600 × *g* for 10 min. The upper hexane layer was transferred to 1.6 mL vials and evaporated to dryness under nitrogen at 60°C. Additional hexane was added to each vial, then again evaporated to dryness. At this stage, samples could be stored at −80°C until later analysis.

Fatty acid methyl esters were analyzed by automated glass capillary gas–liquid chromatography on the Varian model 600 gas chromatograph interfaced with the Varian Vista 402 data system (Varian Instruments, Georgetown, Ontario, Canada), using a fluid silica BP20 capillary column (25 m × 0.25 mm i.d.; Varian) (36). Fatty acid methyl esters were quantitated using flame-ionization detectors. The column was an open tubular glass capillary column (18 m × 0.25 mm i.d.) coated with SP1000/grade AA). Helium was used as a carrier gas at a flow rate of 1.8 mL/min and with an inlet pressure of 80 kPa. The inlet splitter was set at 4 to 1. Samples were injected at 175°C, and after 3 min in the oven the temperature was increased at a rate of 2°C/min for 20 min, and chromatography was complete after 50 min. Fatty acid methyl esters were identified with known standards and by the method of equivalent chain length (37).

Statistics. The results are expressed as mean ± SEM. To determine the effect of dietary rechallenge ("critical period programming"), *t*-tests were performed between the following groups: ScSSc vs. SSSc; SScS vs. ScScS; FcFFc vs. FFFc; and FFcF vs. FcFcF. A statistically significant difference was accepted as $P < 0.05$.

RESULTS

Animals. Refeeding with diets enriched with S, Sc, F, or Fc had no effect on the animals' food consumption or body weight gain (data not shown).

BBM enzyme activity. The BBM invertase activity was enriched 18-fold, and BBM alkaline phosphatase activity was enriched sevenfold, as compared with homogenates (data not shown). No difference in enrichment was noted among the eight diet groups.

Rechallenge with S was associated with an increase in the ileal BBM ratio of alkaline phosphatase/invertase, as compared with animals fed Sc for 9 wk and then fed S for 2 wk (SScS vs. ScScS; 2.1 ± 0.5 vs. 1.0 ± 0.2, respectively, $P < 0.05$, Table 3). Refeeding with Fc resulted in an increased jejunal BBM activity of alkaline phosphatase (FcFFc vs. FFFc; 2058 ± 216 vs. 1304 ± 223, $P < 0.05$), whereas rechallenge with F resulted in a decline in the jejunal BBM activity of alkaline phosphatase (FFcF vs. FcFcF; 1342 ± 178 vs. 2544 ± 465, $P < 0.05$).

BBM lipids. There was no diet effect on BBM cholesterol, but refeeding Fc resulted in increased ($P < 0.05$) ileal BBM total phospholipids (FcFFc vs. FFFc), and rechallenge with F resulted in a decline in the jejunal BBM ratio of phospholipid/cholesterol (FFcF vs. FcFcF) (Table 4).

Refeeding Sc resulted in a decrease in the ileal BBM PC (ScSSc vs. SSSc; 78 ± 11 vs. 176 ± 46, $P < 0.05$, Table 5). In rats rechallenged with Fc, there was an increase in ileal BBM sphingomyelin (FcFFc vs. FFFc; 188 ± 35 vs. 83 ± 14, $P < 0.05$), and PE (195 ± 22 vs. 121 ± 12, $P < 0.05$). There was a corresponding decrease in the ratio of PC/PE (0.5 ± 0.1 vs. 1.0 ± 0.1, $P < 0.05$), as well as an increase in the ratio of sphingomyelin/PC (1.3 ± 0.3 vs. 0.6 ± 0.1, $P < 0.01$).

The major fatty acids in BBM PC and PE were 18:0, 18:2n-6, 16:0, 18:1n-9, and 20:4n-6 (Table 6). Refeeding with different dietary lipids had no effect on the fatty acyl constituents of the jejunal or ileal BBM PC or PE.

DISCUSSION

The intestinal adaptive response to dietary rechallenge depends upon the presence of cholesterol: refeeding S or F has no effect on BBM phospholipids, whereas rechallenge with

TABLE 3
Effect of Dietary Rechallenge on Brush Border Membrane (BBM) Enzyme Markers Using Different Dietary Fatty Acid Unsaturation With or Without Cholesterol Supplementation[a]

		Rechallenge with Sc		Rechallenge with S		Rechallenge with Fc		Rechallenge with F	
Marker		Control SSSc	Rechallenge ScSSc	Control ScScS	Rechallenge SScS	Control FFFc	Rechallenge FcFFc	Control FcFcF	Rechallenge FFcF
Mucosal scrapings	JEJ	30 ± 3	33 ± 3	36 ± 2	30 ± 1*	27 ± 1	26 ± 2	22 ± 2	28 ± 2
(mg/cm length)	ILE	24 ± 2	28 ± 2	31 ± 2	25 ± 2*	23 ± 3	24 ± 2	21 ± 2	21 ± 1
Protein	JEJ	1.33 ± 0.21	1.33 ± 0.17	1.31 ± 0.23	1.43 ± 0.20	1.17 ± 0.13	1.16 ± 1.15	1.49 ± 0.12	1.60 ± 0.14
(mg/g wet weight)	ILE	1.18 ± 0.12	1.06 ± 0.26	1.26 ± 0.22	1.19 ± 0.15	1.40 ± 0.17	1.10 ± 0.12	1.55 ± 0.31	1.37 ± 0.18
Alkaline phosphatase	JEJ	1805 ± 274	2285 ± 352	1580 ± 411	1263 ± 293	1304 ± 233	2058 ± 216*	2544 ± 465	1342 ± 178*
(U/g protein)	ILE	550 ± 193	692 ± 183	396 ± 81	502 ± 176	612 ± 138	812 ± 177	529 ± 124	690 ± 215
Invertase	JEJ	651 ± 84	848 ± 108	754 ± 119	700 ± 44	594 ± 120	645 ± 81	751 ± 99	734 ± 95
(U/g protein)	ILE	456 ± 76	407 ± 82	515 ± 96	522 ± 138	468 ± 155	343 ± 52	309 ± 45	360 ± 62
Alkaline phosphatase/	JEJ	2.1 ± 0.3	2.8 ± 0.5	1.8 ± 0.2	1.8 ± 0.3	2.6 ± 0.6	3.4 ± 0.6	2.8 ± 0.4	2.3 ± 0.3
invertase	ILE	2.2 ± 0.4	1.7 ± 0.3	1.0 ± 0.2	2.1 ± 0.5*	1.7 ± 0.4	2.0 ± 0.8	2.3 ± 0.5	1.9 ± 0.5

[a]Mean ± SEM; *$P < 0.05$, ScSSc vs. SSSc; SScS vs. ScScS; FcFFc vs. FFFc; FFcF vs. FcFcF. The presence of NADPH-cytochrome C reductase and DNA were not detected in the BBM preparations from any diet group. Na^+K^+-ATPase activity was low in the BBM preparations for all diet groups. On average, invertase activity was enriched 18× and alkaline phosphatase activity enriched 7×; JEJ, jejunum; IEL, ileum; SSSc, 9 wk S/2 wk Sc; ScSSc, 2 wkSc/7 wk S/2 wk Sc; ScScS, 9 wk Sc/2 wk S; SScS, 2 wk S/7 wk Sc/2 wk S; FFFc, 9 wk F/2 wk Fc; FcFFc, 2 wk Fc/7 wk F/2 wk Fc; FcFcF, 9 wk Fc/2 wk F; FFcF, 2 wk F/7 wk Fc/2 wk F. See Table 1 for other abbreviations.

Sc or Fc results in modifications of BBM phospholipids (Table 5). Dietary rechallenge with Fc enhances jejunal BBM alkaline phosphatase activity as compared with animals fed only F and later challenged with Fc. The reverse was also true: dietary rechallenge with F reduced jejunal BBM alkaline phosphatase activity as compared with animals fed Fc and later challenged with F. Ileal BBM total phospholipids are increased in animals rechallenged with Fc, and reduced when rechallenged with F, owing to changes in sphingomyelin and PE content. Although the expected changes in BBM fatty acid composition are observed with feeding fish oil vs. saturated fatty acids, the presence or absence of cholesterol in the diet does not alter BBM phospholipid fatty acid composition with dietary rechallenge. This observation is consistent with the results of previous studies in which BBM cholesterol content appears to be relatively resistant to modification in a variety of models of intestinal adaptation (4–6,38). Thus, cholesterol must be present in the diet for the rechallenge effect on BBM phospholipids to be observed.

The same feeding program used in this study is associated with alterations in intestinal transport (20). In most previous studies of intestinal adaptation of dietary lipid alterations, the modified uptake of nutrients is associated with changes in the BBM phospholipid fatty acid composition (39–41). While diet rechallenge was associated with changes in BBM phospholipids, this was seen only in the ileum and only when cholesterol was present. Since rechallenge with S or F also results in adaptations of nutrient uptake in the jejunum, there must be some mechanism other than BBM phospholipids to explain the changes in uptake. Other factors considered were BBM phospholipid fatty acids (Tables 6–9), food intake, and body weight gain, but these were also not explanations.

TABLE 4
Effect of Dietary Rechallenge on Brush Border Membrane Lipid Composition Using Different Dietary Fatty Acid Unsaturation With or Without Cholesterol Supplementation[a]

		Rechallenge with Sc		Rechallenge with S		Rechallenge with Fc		Rechallenge with F	
Lipid (nmoles/mg protein)		Control SSSc	Rechallenge ScSSc	Control ScScS	Rechallenge SScS	Control FFFc	Rechallenge FcFFc	Control FcFcF	Rechallenge FFcF
Total phospholipids	JEJ	324 ± 43	289 ± 43	453 ± 27	439 ± 79	508 ± 82	379 ± 87	569 ± 56	523 ± 52
	ILE	371 ± 33	334 ± 34	420 ± 77	347 ± 87	332 ± 52	525 ± 53*	517 ± 65	317 ± 52
Total cholesterol	JEJ	216 ± 28	212 ± 23	207 ± 32	167 ± 21	214 ± 32	204 ± 21	158 ± 23	172 ± 18
	ILE	239 ± 23	212 ± 44	177 ± 17	169 ± 17	196 ± 29	183 ± 20	194 ± 17	158 ± 16
Phospholipid/	JEJ	1.7 ± 0.3	1.4 ± 0.4	2.7 ± 0.3	2.2 ± 0.5	2.4 ± 0.3	1.7 ± 0.3	3.8 ± 0.2	2.3 ± 0.3*
cholesterol	ILE	2.0 ± 0.3	1.5 ± 0.3	3.0 ± 0.6	2.2 ± 0.4	2.3 ± 0.5	2.6 ± 0.4	2.9 ± 0.4	2.6 ± 0.4

[a]Mean ± SEM; *$P < 0.05$, ScSSc vs. SSSc; SScS vs. ScScS; FcFFc vs. FFFc; FFcF vs. FcFcF. See Tables 1 and 3 for abbreviations. See Table 3 for diet and week distribution.

TABLE 5
Effect of Dietary Rechallenge on Brush Border Membrane Phospholipids Using Different Dietary Fatty Acid Unsaturation With or Without Cholesterol Supplementation[a]

Phospholipid (nmoles/mg protein)		Rechallenge with Sc		Rechallenge with S		Rechallenge with Fc		Rechallenge with F	
		Control SSSc	Rechallenge ScSSc	Control ScScS	Rechallenge SScS	Control FFFc	Rechallenge FcFFc	Control FcFcF	Rechallenge FFcF
Phosphatidylcholine	JEJ	142 ± 34	101 ± 26	162 ± 34	125 ± 28	182 ± 23	182 ± 40	234 ± 22	189 ± 39
	ILE	176 ± 46	78 ± 11*	102 ± 26	160 ± 43	127 ± 24	124 ± 16	144 ± 16	159 ± 41
Sphingomyelin	JEJ	67 ± 13	65 ± 13	139 ± 30	88 ± 20	90 ± 21	84 ± 16	103 ± 16	100 ± 20
	ILE	107 ± 31	82 ± 17	156 ± 34	146 ± 35	83 ± 14	188 ± 35*	110 ± 22	92 ± 40
Phosphatidylethanolamine	JEJ	117 ± 19	95 ± 18	243 ± 43	161 ± 32	203 ± 29	179 ± 39	261 ± 41	225 ± 31
	ILE	190 ± 34	123 ± 26	183 ± 34	159 ± 50	121 ± 12	195 ± 22*	194 ± 35	148 ± 42
Phosphatidylserine	JEJ	8 ± 3	8 ± 5	9 ± 3	9 ± 5	8 ± 2	7 ± 5	19 ± 7	5 ± 3
	ILE	15 ± 6	10 ± 3	17 ± 9	14 ± 6	12 ± 4	9 ± 5	10 ± 4	26 ± 14
Phosphatidylinositol	JEJ	19 ± 10	16 ± 6	18 ± 5	14 ± 3	26 ± 5	29 ± 8	17 ± 4	28 ± 9
	ILE	9 ± 3	11 ± 4	18 ± 8	27 ± 10	7 ± 2	17 ± 5	28 ± 10	16 ± 5
Sphingomyelin/ phosphatidylcholine	JEJ	0.7 ± 0.2	1.0 ± 0.3	0.9 ± 0.2	0.9 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
	ILE	0.8 ± 0.3	1.1 ± 0.2	1.0 ± 0.2	0.9 ± 0.1	0.6 ± 0.1	1.3 ± 0.3*	0.9 ± 0.3	0.8 ± 0.4
Phosphatidylcholine/ phosphatidylethanolamine	JEJ	1.2 ± 0.2	1.3 ± 0.4	0.7 ± 0.1	0.8 ± 0.2	1.0 ± 0.1	0.8 ± 0.1	1.2 ± 0.2	0.9 ± 0.1
	ILE	1.0 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	1.0 ± 0.1	0.5 ± 0.1*	0.8 ± 0.2	0.8 ± 0.1

[a]Mean ± SEM; *$P < 0.05$, ScSSc vs. SSSc; SScS vs. ScScS; FcFFc vs. FFFc; FFcF vs. FcFcF. See Tables 1 and 3 for abbreviations. See Table 3 for diet and week distribution.

TABLE 6
Effect of Dietary Rechallenge on Jejunal Brush Border Membrane Phosphatidylcholine Fatty Acid Composition Using Different Dietary Fatty Acid Unsaturation With or Without Cholesterol Supplementation[a]

Fatty acid (% of total fatty acids)	Rechallenge with Sc		Rechallenge with S		Rechallenge with Fc		Rechallenge with F	
	Control SSSc	Rechallenge ScSSc	Control ScScS	Rechallenge SScS	Control FFFc	Rechallenge FcFFc	Control FcFcF	Rechallenge FFcF
16:0	14.8 ± 1.41	10.9 ± 2.62	10.3 ± 1.62	10.1 ± 1.95	9.5 ± 1.40	9.8 ± 2.61	9.5 ± 1.88	11.3 ± 2.85
16:1n-7	0.3 ± 0.12	0.4 ± 0.09	0.1 ± 0.06	0.1 ± 0.05	0.5 ± 0.15	1.0 ± 0.21	0.5 ± 0.09	0.7 ± 0.23
18:0	34.1 ± 1.28	33.4 ± 2.09	33.9 ± 1.52	32.2 ± 0.99	32.0 ± 1.25	33.2 ± 1.12	34.5 ± 0.65	34.3 ± 1.16
18:1n-9	10.2 ± 0.53	10.9 ± 0.35	7.6 ± 0.31	8.1 ± 0.58	4.8 ± 0.15	4.5 ± 0.19	4.1 ± 0.24	3.8 ± 0.19
18:1n-7	1.1 ± 0.06	1.3 ± 0.10	0.9 ± 0.17	0.9 ± 0.17	1.8 ± 0.08	1.7 ± 0.10	1.7 ± 0.08	1.8 ± 0.09
18:2n-6	24.9 ± 1.18	21.2 ± 1.21	26.6 ± 1.20	23.8 ± 2.08	12.8 ± 0.34	13.2 ± 0.79	12.8 ± 0.40	12.9 ± 0.77
18:3n-3	0.1 ± 0.04	0.2 ± 0.08	0.1 ± 0.04	0.1 ± 0.05	0.2 ± 0.06	0.1 ± 0.05	0.1 ± 0.02	0.1 ± 0.06
20:3n-6	1.3 ± 0.25	1.5 ± 0.18	1.4 ± 0.16	1.8 ± 0.39	0.5 ± 0.09	0.5 ± 0.04	0.6 ± 0.10	0.6 ± 0.11
20:4n-6	7.8 ± 0.41	9.6 ± 1.27	10.3 ± 0.78	13.2 ± 1.86	6.5 ± 0.36	6.7 ± 0.61	7.3 ± 0.53	7.3 ± 0.64
20:5n-3	0.1 ± 0.05	0.6 ± 0.25	0.2 ± 0.11	0.5 ± 0.11	19.7 ± 0.70	19.3 ± 1.34	19.4 ± 0.94	17.7 ± 1.53
22:4n-6	0.2 ± 0.06	0.7 ± 0.19	0.4 ± 0.8	0.7 ± 0.8	0.4 ± 0.11	0.5 ± 0.15	0.4 ± 0.07	0.2 ± 0.08
22:5n-6	0.3 ± 0.10	1.0 ± 0.29	0.7 ± 0.15	0.9 ± 0.25	0.2 ± 0.07	0.2 ± 0.08	0.3 ± 0.08	0.1 ± 0.04
22:5n-3	0.1 ± 0.10	n.d.	trace	0.1 ± 0.06	0.7 ± 0.09	0.4 ± 0.08	0.7 ± 0.10	0.7 ± 0.14
22:6n-3	0.3 ± 0.07	0.4 ± 0.10	0.4 ± 0.04	0.5 ± 0.07	3.8 ± 0.25	3.5 ± 0.43	3.5 ± 0.32	3.5 ± 0.56
Total saturates (S)	51.2 ± 1.39	48.1 ± 1.20	48.7 ± 1.59	45.5 ± 1.18	43.6 ± 0.71	45.5 ± 1.63	46.1 ± 1.82	48.2 ± 2.61
Total monounsaturates (M)	12.8 ± 0.61	14.8 ± 0.29	9.8 ± 0.35	11.2 ± 0.62	9.0 ± 0.46	8.7 ± 0.55	7.6 ± 0.34	7.7 ± 0.30
Total polyunsaturates (P)	35.8 ± 1.38	36.6 ± 1.09	41.2 ± 1.40	42.8 ± 0.67	47.0 ± 1.03	45.6 ± 1.37	46.0 ± 1.68	43.9 ± 2.46
Total n-9	10.7 ± 0.49	12.0 ± 0.42	8.3 ± 0.27	9.6 ± 0.60	6.3 ± 0.47	5.6 ± 0.44	5.0 ± 0.28	4.9 ± 0.34
Total n-7	2.0 ± 0.10	2.8 ± 0.22	1.7 ± 0.28	1.8 ± 0.28	2.6 ± 0.24	3.0 ± 0.14	2.5 ± 0.11	2.8 ± 0.20
Total n-6	35.3 ± 1.38	35.4 ± 1.13	40.5 ± 1.31	41.6 ± 0.68	22.4 ± 0.50	22.2 ± 0.61	22.2 ± 0.48	21.7 ± 0.44
Total n-3	0.5 ± 0.11	1.2 ± 0.34	0.7 ± 0.12	1.2 ± 0.18	24.6 ± 0.89	23.4 ± 1.54	23.8 ± 1.31	22.2 ± 2.16
M/S	0.3 ± 0.02	0.3 ± 0.01	0.2 ± 0.01	0.3 ± 0.02	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.01	0.2 ± 0.01
P/S	0.7 ± 0.04	0.8 ± 0.04	0.9 ± 0.05	1.0 ± 0.04	1.1 ± 0.04	1.0 ± 0.06	1.0 ± 0.07	0.9 ± 0.10
Unsaturation index	105 ± 4	119 ± 4	121 ± 5	134 ± 6	197 ± 5	190 ± 8	192 ± 9	182 ± 13

[a]Mean ± SEM. None of these differences were statistically significant, $P < 0.05$; n.d., not detected; trace (<0.05%). See Tables 1 and 3 for abbreviations. See Table 3 for diet and week distribution.

Furthermore, the lack of clear association of BBM lipid compositional changes and BBM nutrient transport indicates that there must be a diet-associated mechanism other than villus morphology, crypt cell production rate, enterocyte turnover time, or distribution of transporters along the villus (42). A possible explanation for the lack of expected association between transport and BBM lipid compositional changes in response to alterations in dietary lipids would be to recognize the heterogeneity of distribution of transporters along the villus, with most transport occurring in the upper third of the villus (42), and with variations in BBM phospholipids along the villus (43,44). In this study, we measured phospholipids and fatty acyl constituents in BBM from enterocytes pooled from along the villus. It is possible that there were diet-associated alterations in BBM lipids in only a portion of the enterocytes from along the villus, and that by measuring fatty acids and phospholipids from the homogenate rather than from portions along the villus, we were unable to detect a membrane compositional change in a portion of cells that was responsible for altered nutrient uptake. This possibility is supported by the finding that the activity of enterocyte microsomal membrane fatty acid and phospholipid metabolic enzymes varies along the villus, as does the enterocyte microsomal membrane composition of fatty acids and phospholipids (Keelan, M., Clandinin, M.T., and Thomson, A.B.R., 1997, unpublished observations). Finally, it has been recognized recently that protein-mediated processes as well as passive permeation participate in the BBM uptake of lipids (45,46), so that diet-associated alterations in lipid uptake may be due only in part to changes in BBM lipids, lipophilic properties, and therefore passive permeation.

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Facile and General Method for Preparation of (*E*)-4-Hydroxy-2-alkenals

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ABSTRACT: A facile one-pot synthesis of (*E*)-4-hydroxy-2-alkenals such as (*E*)-4-hydroxy-2-nonenal, (*E*)-4-hydroxy-2-heptenal, and (*E*)-4-hydroxy-2-hexenal was achieved from the corresponding (2*E*,4*E*)-2,4-alkadienals by reduction–oxygenation with molecular oxygen and triethylsilane in the presence of cobalt(II) porphyrin as a catalyst followed by treatment with trimethylphosphite.

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Lipid peroxidation in biological systems produces many degradation products, including (*E*)-4-hydroxy-2-alkenals, which exhibit biological activity including cytotoxicity and genotoxicity (1,2). (*E*)-4-Hydroxy-2-nonenal (HNE) and (*E*)-4-hydroxy-2-hexenal (HHE) are major aldehydes derived from n-6 and n-3 unsaturated fatty acids, respectively. The synthesis of pure (*E*)-4-hydroxy-2-alkenals is required to study their biological behaviors. Consequently several synthetic efforts have been published (3–8).

Recently, we found that α,β,γ,δ-unsaturated carbonyl compounds were converted into γ-hydroxy-α,β-unsaturated carbonyl compounds regioselectively using cobalt(II) porphyrin-catalyzed reduction–oxygenation followed by reduction *in situ* (9). Using this method, we describe herein a facile and general method for a one-pot preparation of (*E*)-4-hydroxy-2-alkenals from (2*E*,4*E*)-2,4-alkadienals.

MATERIALS AND METHODS

Materials. (2*E*,4*E*)-2,4-Nonadienal, (2*E*,4*E*)-2,4-heptadienal, and (2*E*,4*E*)-2,4-hexadienal were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and freshly distilled under reduced pressure. 2-Propanol and dichloromethane (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were distilled from calcium hydride. Trimethylphosphite was purchased from Wako Pure Chemical Industries, Ltd. Triethylsilane and chlorotriethylsilane were purchased from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). 2-Propan(ol-d), lithium aluminum deuteride, and [5,10,15,20-tetraphenylporphinato)cobalt(II) (abbreviated as Co(tpp)] were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). {[5,10,15,20-Tetrakis(2,6-dichlorophenyl)porphinato]cobalt(II) (abbreviated as Co(tdcpp)} was prepared from commercially available 5,10,15,20-tetrakis(2,6-dichlorophenyl)porphine (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) by the insertion of cobalt ion using a method described in the literature (10). Triethyldeuterosilane was obtained by the reaction of chlorotriethylsilane with lithium aluminum deuteride according to a described method (11). Column chromatography was carried out using BW-300 purchased from Fuji Silysia Co., Ltd. (Aichi, Japan).

Typical procedure for synthesis of HNE. To a dichloromethane solution (5 mL) of (2*E*,4*E*)-2,4-nonadienal (277 mg, 2 mmol) and Co(tdcpp) (1.9 mg, 0.002 mmol) in a 50-mL Kjeldahl flask equipped with a three-way stopcock were added 5 mL of 2-propanol. The atmosphere in the flask was replaced with oxygen by bubbling into the solution for 5 min and then an oxygen balloon was attached to the flask through the three-way stopcock. After triethylsilane (0.35 mL, 2.2 mmol) was added to the solution at 28°C, the mixture was stirred at 28°C for 3 h, followed by treatment with trimethylphosphite (0.3 mL, 2.6 mmol) at 0°C for 2 h at room temperature. After the solvent was removed under reduced pressure, the residue was purified by silica gel column chromatography with *n*-hexane/ethyl acetate (16:1–4:1, vol/vol) to afford oily HNE (180 mg, 57.7%).

Synthesis of (E)*-4-hydroxy-2-[5-$^2H_1$]nonenal.* (*E*)-4-Hydroxy-2-[5-$^2H_1$]nonenal ([^{2}H]HNE) was prepared in 62.4% yield by the same procedure as described above except that triethyldeuterosilane and 2-propan(ol-d) were used instead of triethylsilane and 2-propanol.

Derivatization to 2,4-dinitrophenylhydrazone. To a solution of HNE (156 mg, 1 mmol) in 2 mL of ethanol were added 20 mL of 2,4-dinitrophenylhydrazine reagent, prepared from 2,4-dinitrophenylhydrazine (2 g), 98% H_2SO_4 (5 mL), H_2O (15 mL), and ethanol (20 mL). The mixture was stirred for 30 min at room temperature, and then the orange precipitate was collected by filtration followed by washing with 10 mL of 50% aqueous ethanol. The precipitate was then dried *in vacuo* to give 2,4-dinitrophenylhydrazone in a quantitative yield.

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Abbreviations: Co(tdcpp), [5,10,15,20-tetrakis(2,6-dichlorophenyl)porphinato]cobalt(II); Co(tpp), (5,10,15,20-tetraphenylporphinato)cobalt(II); EI-MS, electron ionization-mass spectrometry; HHE, (*E*)-4-hydroxy-2-hexenal; [^{2}H]HNE, (*E*)-4-hydroxy-2-[5-$^2H_1$]nonenal; HNE, (*E*)-4-hydroxy-2-nonenal; NMR, nuclear magnetic resonance.

Spectral analyses. ^{1}H (250 MHz) and ^{13}C (63 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC-250P (Rheinstetten, Germany) in $CDCl_3$ with tetramethylsilane ($\delta = 0.00$) as an internal standard. Infrared (IR) spectra were measured in $CHCl_3$ with a Hitachi IR 270-30 (Tokyo, Japan) spectrometer. Electron ionization–mass spectrometry (EI–MS) was effected on a Hitachi M-2000AM mass spectrometer operating at 70 eV. Melting points were measured using a Yanako MP-500D (Kyoto, Japan).

HNE: ^{1}H NMR: δ_{ppm} 0.90 (*t*, *J* = 6.3 Hz, 3H), 1.31–1.65 (*m*, 8H), 2.30 (*brs*, 1H), 4.44 (*dtd*, *J* = 1.6, 4.7, and 6.5 Hz, 1H), 6.31 (*ddd*, *J* = 1.6, 7.9, and 15.6 Hz, 1H), 6.85 (*dd*, *J* = 4.7 and 15.6 Hz, 1H), 9.58 (*d*, *J* = 7.9 Hz, 1H). ^{13}C NMR: δ_{ppm} 14.01, 22.54, 24.91, 31.62, 36.46, 71.11, 130.61, 159.36, 193.79. IR: ν (cm^{-1}) 3620, 3500, 3050–2750, 1690, 1640, 1470, 1390. *2,4-Dinitrophenylhydrazone:* EI–MS: *m/z* 336 (M^+), m.p. 154–155°C. Calcd. for $C_{15}H_{20}N_4O_5$ (%): C: 53.56, H: 5.99, N: 16.65; found: C: 53.50, H: 5.98, N: 16.63.

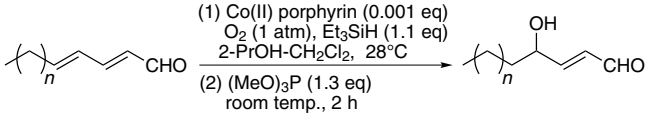
(E)-4-Hydroxy-2-heptenal: ^{1}H NMR: δ_{ppm} 0.96 (*t*, *J* = 7.0 Hz, 3H), 1.40–1.69 (*m*, 4H), 2.58 (*brs*, 1H), 4.45 (*dtd*, *J* = 1.6, 4.8, and 6.2 Hz, 1H), 6.31 (*ddd*, *J* = 1.6, 7.9, and 15.6 Hz, 1H), 6.86 (*dd*, *J* = 4.8 and 15.6 Hz, 1H), 9.56 (*d*, *J* = 7.9 Hz, 1H). ^{13}C NMR: δ_{ppm} 13.85, 18.48, 38.47, 70.76, 130.48, 159.72, 193.96. IR: ν (cm^{-1}) 3650, 3500, 3050–2750, 1700, 1640, 1470, 1390. *2,4-Dinitrophenylhydrazone:* EI–MS: *m/z* 308 (M^+), m.p. 179–180°C. Calcd. for $C_{13}H_{16}N_4O_5$ (%): C: 50.65, H: 5.23, N: 18.17; found: C: 50.67, H: 5.23, N: 18.03.

HHE: ^{1}H NMR: δ_{ppm} 0.98 (*t*, *J* = 7.4 Hz, 3H), 1.59–1.75 (*m*, 2H), 2.63 (*brs*, 1H), 4.38 (*dtd*, *J* = 1.5, 4.7, and 6.7 Hz, 1H), 6.31 (*ddd*, *J* = 1.5, 7.9, and 15.7 Hz, 1H), 6.85 (*dd*, *J* = 4.7 and 15.7 Hz, 1H), 9.57 (*d*, *J* = 7.9 Hz, 1H). ^{13}C NMR: δ_{ppm} 9.50, 29.45, 72.22, 130.82, 159.27, 193.90. IR: ν (cm^{-1}) 3620, 3500, 3050–2780, 1700, 1640, 1480, 1390. *2,4-Dinitrophenylhydrazone:* EI–MS: *m/z* 294 (M^+), m.p. 196–197°C. Calcd. for $C_{12}H_{14}N_4O_5$ (%): C: 48.98, H: 4.80, N: 19.04; found: C: 48.94, H: 4.81, N: 18.82.

[^{2}H]HNE: ^{1}H NMR: δ_{ppm} 0.90 (*t*, *J* = 6.5 Hz, 3H), 1.31–1.61 (*m*, 7H), 2.12 (*brs*, 1H), 4.44 (*ddd*, *J* = 1.5, 5.0, 6.4 Hz, 1H), 6.31 (*ddd*, *J* = 1.5, 7.9, and 15.6 Hz, 1H), 6.83 (*dd*, *J* = 5.0 and 15.6 Hz, 1H), 9.58 (*d*, *J* = 7.9 Hz, 1H). ^{13}C NMR: δ_{ppm} 13.91, 22.46, 24.72, 31.53, 35.76, 36.07, 36.37, 71.03, 130.61, 158.99, 193.54. IR: ν (cm^{-1}) 3620, 3500, 3030–2750, 1690, 1640, 1470, 1380. *2,4-Dinitrophenylhydrazone:* EI–MS: *m/z* 337 (M^+), m.p. 146–147°C. Calcd. for $C_{15}D_1H_{19}N_4O_5$ (%): C: 53.41, H: 5.97, N: 16.61; found: C: 52.77, H: 5.93, N: 16.40.

RESULTS AND DISCUSSION

Gardner *et al.* (8) reported a two-step synthesis of HNE starting from 3(*Z*)-nonenal. This method described a facile and efficient synthesis of HNE in comparison with previously described procedures (3–7). Recently, we found that α,β,γ,δ-unsaturated carbonyl compounds were converted into γ-hydroxy-α,β-unsaturated carbonyl compounds regioselectively using a cobalt(II) porphyrin-catalyzed reduction–oxygenation followed by reduction *in situ* (9). We expected that various (*E*)-4-hydroxy-2-alkenals could be synthesized in one pot from commercially available (2*E*,4*E*)-2,4-alkadienals by reduction–oxygenation. In practice, (2*E*,4*E*)-2,4-alkadienals were made to react with catalytic amounts of cobalt(II) porphyrin (0.001 eq) and triethylsilane (1.1 eq) under 1 atm of oxygen followed by treatment of trimethylphosphite (1.3 eq) in one pot (Scheme 1). The results are summarized in Table 1. When Co(tpp) was used as the catalyst, yields of (*E*)-4-hydroxy-2-alkenals were low (Entries 1–3), because the Co(tpp) had decomposed within 1 h, before reaction was complete. In contrast, when Co(tdcpp) was used instead of Co(tpp), (*E*)-4-hydroxy-2-alkenals were obtained in good yields (Entries 4–6), since the Co(tdcpp) was stable for several hours under the given reaction conditions.



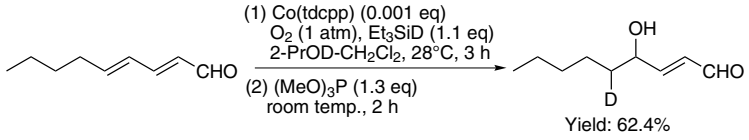
SCHEME 1

Labeled HNE is important for pharmacodynamic and metabolic studies. Bravais *et al.* (12) have already reported synthesis of tritium- and deuterium-labeled (*E*)-4-hydroxy-2-nonenal-diethylacetal, but this method required a multistep procedure. Therefore, we tried one-pot synthesis of the labeled HNE using a Co(tdcpp)-catalyzed reduction–oxygenation. (2*E*,4*E*)-2,4-Nonadienal was allowed to react with oxygen and triethyldeuterosilane and 2-propan(ol-d) under conditions as already described, to give the deuterium-labeled HNE after purification by column chromatography (Scheme 2). The product showed the following ^{1}H NMR chemical shifts: ppm (δ), and (multiplicity, coupling constant in Hz, number of protons, and carbon assignments): 4.44 (*ddd*, *J* = 1.5, 5.0, 6.4 Hz, 1H, C-4), 6.31 (*ddd*, *J* = 1.5, 7.9, and 15.6 Hz, 1H, C-2), 6.83 (*dd*, *J* = 5.0 and 15.6 Hz, 1H, C-3), 9.58 (*d*, *J* = 7.9 Hz, 1H, C-1). Its 2,4-DNPH derivative showed molecular ion (*m/z*,

TABLE 1
Synthesis of (*E*)-4-Hydroxy-2-alkenals from (2*E*,4*E*)-2,4-Alkadienals

Entry	Alkadienal (*n* =)	Co(II) porphyrin	Time[a] (h)	Yield[b] (%)
1	3	Co(tpp)	1	38.7
2	1	Co(tpp)	1	27.6
3	0	Co(tpp)	1	33.9
4	3	Co(tdcpp)	3	57.7
5	1	Co(tdcpp)	3	53.7
6	0	Co(tdcpp)	3	60.8

[a]Reaction time for reduction–oxygenation step.
[b]Isolated yield by silica gel column chromatography. Abbreviations: Co(tdcpp): [5,10,15,20-tetrakis(2,6-dichlorophenyl)porphinato]cobalt(II); Co(tpp): (5,10,15,20-tetraphenylporphinato)cobalt(II).



SCHEME 2

EI–MS): 337 (M^+). It was confirmed that (*E*)-4-hydroxy-2-[5-$^2H_1$]nonenal was produced in a 62.4% yield. This method may be applicable for the preparation of tritium-labeled HNE, (*E*)-4-hydroxy-2-[5-$^3H_1$]nonenal.

We have described a one-pot method synthesis of HNE derivatives from (2*E*,4*E*)-2,4-alkadienals using cobalt(II) porphyrin-catalyzed reduction–oxygenation.

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Lipase-Catalyzed Monoesterification of 1-*O*-Hexadecylglycerol in Organic Solvents

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ABSTRACT: A simple method is presented to esterify 1-*O*-hexadecyl-*rac*-glycerol using lipases in different organic solvents. The following fatty acids were used: $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$. Monoesterification was achieved by using a limiting amount of the fatty acid. Both the 1-*O*-hexadecyl-3-*O*-acylglycerol and the 2-*O*-acylglycerol were obtained in a total yield of 75% and a ratio of 7:1 in dichloromethane after 3 d. Chromatographic data for the monoesters, useful for the identification of the natural products, are given (gas–liquid chromatography, thin-layer chromatography, reverse-phase thin-layer chromatography). The structure was confirmed by a chemical synthesis of 1-*O*-hexadecyl-2-*O*-hexadecanoylglycerol. The 3-*O*-glyceride was also formed by acyl migration, as the minor component. The monoesters were separated by column chromatography and characterized by ^1H and ^{13}C nuclear magnetic resonance spectra.

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Glyceryl ether lipids of the 1-*O*-alkyl-2-*O*-acylglycerol type were described as components of free or protein-linked glycosylphosphatidylinositols (GPI) (1). In particular, 1-*O*-hexadecyl-2-*O*-hexadecanoylglycerol was identified in GPI of epimastigote forms of *Trypanosoma cruzi* (2), in the GPI anchor of the 1G7-antigen of metacyclic forms of *T. cruzi* (3), and in the sialic acid acceptor mucins of this parasite (4). Interestingly, in the latter, the 1-*O*-alkyl-2-*O*-acylglycerol was mostly replaced by ceramide when epimastigotes differentiate to the infective metacyclic forms.

On the other hand, structural analysis of the phosphatidylinositol lipid class in *T. cruzi* epimastigotes showed that the alkylacyl species is more abundant than the diacylglycerol lipids. Hexadecylglycerol was esterified with the $C_{18:1}$ and $C_{18:2}$ fatty acids, identified by gas–liquid chromatography (GLC)–mass spectrometry (5). In the infective trypomastigote forms of *T. cruzi,* hexadecylglycerol esterified with the $C_{18:0}$ fatty acid was detected by *in vivo* incorporation of [^3H] palmitic acid (6).

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Abbreviations: GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance; RPTLC, reverse-phase thin-layer chromatography; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

In connection with our studies on the structure of GPI, standards of alkylacylglycerols were required for capillary GLC and thin-layer chromatography (TLC) analyses.

Lipases in organic solvents have been widely used for the synthesis or hydrolysis of fatty acid esters (7,8). Enzymatic esterification of 1-*O*-octadecyl-*rac*-glycerol with oleic acid in the presence of organic solvents using the lipase from *Candida cylindracea* was investigated. A considerable proportion of the diester was obtained when an excess of oleic acid was used (9). Diesterification of 1-*O*-alkyl-*sn*-glycerols with eicosapentaenoic acid and docosahexaenoic acid, respectively, by lipase was reported (10).

In the present work, 1-*O*-hexadecyl-*rac*-glycerol was esterified with $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$ by using the nonregiospecific lipase from *C. cylindracea* (11) or the lipase from *Pseudomonas* sp. (7). A limiting amount of fatty acid was used in order to obtain monoesterification of the hexadecylglycerol. Furthermore, the monopalmitoyl derivatives were chemically synthesized and characterized by the nuclear magnetic resonance (NMR) spectra.

MATERIALS AND METHODS

Materials. Lipases from *C. cylindracea* (new name, *C. rugosa*) Type VII (Sigma, St. Louis, MO) and from *Pseudomonas* sp. Type XIII (Sigma) were used. All chemicals were of analytical grade. An incubator shaker (New Brunswick Science INNOVA 4000, Edison, NJ) at 200 rpm was used.

Analytical methods. TLC was performed using silica-gel 60 (Merck, Darmstadt, Germany) with the following solvent systems: (i) hexane/ethyl acetate (4:1, vol/vol); (ii) toluene/ethyl acetate (20:1, vol/vol). Reverse-phase TLC (RPTLC) was performed on RP-18 F_{254s} (Merck) using chloroform/methanol/water (40:100:3, by vol) as solvent. Compounds were detected by spraying the plates with 10% H_2SO_4/0.04 M $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$/3 mM $Ce(SO_4)_2$ and heating to 200°C.

The lipids as trimethylsilyl derivatives were analyzed with a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) with a flame-ionization detector and nitrogen as the carrier gas. An HP-1 capillary column (12 m × 0.22 mm) was used.

TABLE 1
Chromatographic Data of Monoesters of 1-*O*-Hexadecylglycerol

Monoester of 1-*O*-hexadecylglycerol[a]	GLC rt (min)[b]	TLC (R_f)[c]	RPTLC (R_f)[d]
2-*O*-$C_{14:0}$	13.213	0.50	0.31
3-*O*-$C_{14:0}$	13.565	0.63	0.31
2-*O*-$C_{16:0}$	15.196	0.50	0.26
3-*O*-$C_{16:0}$	15.671	0.63	0.26
2-*O*-$C_{18:2}$	17.470	n.d.	0.30
3-*O*-$C_{18:2}$	18.115	n.d.	0.30
2-*O*-$C_{18:1}$	17.475	n.d.	0.27
3-*O*-$C_{18:1}$	18.140	n.d	0.27
2-*O*-$C_{18:0}$	17.798	0.50	0.23
3-*O*-$C_{18:0}$	18.455	0.63	0.23

[a]2-*O*-$C_{14:0}$ = 2-*O*-myristoyl-1-*O*-hexadecylglycerol, etc.
[b]Gas–liquid chromatography (GLC) of the trimethylsilyl derivatives using an HP-1 capillary column (0.2 mm × 12 m). The oven temperature program was 245°C (1 min) to 255°C at 3°C/min and to 320°C at 9°C/min. Retention time, rt.
[c]Thin-layer chromatography (TLC) on silica gel 60 (Merck, Darmstadt, Germany) using the solvent system hexane/ethyl acetate (4:1, vol/vol). Not detected, n.d.
[d]Reverse-phase thin-layer chromatography (RPTLC) on RP-18 F254s (Merck) using the solvent chloroform/ethanol/water (40:100:3, by vol).

The temperature was maintained at 245°C for 1 min, and then increased to 255°C at 3°C/min and then to 320°C at 9°C/min. Quantitative analyses were performed using 5-α-cholestane as internal standard. Aliquots of the reaction products were taken to dryness with a nitrogen stream, and trimethylsilylation was performed with 20–50 µL of Sylon HTP (Supelco, Bellefonte, PA) by heating at 70°C for 10 min.

The products were characterized by their ^{1}H and ^{13}C NMR spectra using a Bruker AC200 spectrometer (Karlsruhe, Germany.

Column purification of the alkylacyl products. The products of the reaction were purified by column chromatography on Unisil (200–325 mesh; Clarkson Chemical Co., South Williamsport, PA), eluting with hexane/ethyl acetate (9:1, vol/vol). The purification was monitored by TLC.

*Esterification of 1-*O*-hexadecylglycerol.* A suspension of 0.1 mmol of 1-*O*-hexadecyl-*rac*-glycerol, 0.05 mmol of myristic, palmitic, stearic, oleic and linoleic acid, respectively, and 1 mg of lipase from *C. cylindracea* or from *Pseudomonas* sp. in 2 mL of organic solvent was stirred at 37°C. The progress of the reaction was monitored by TLC and GLC. For chromatographic data, see Table 1.

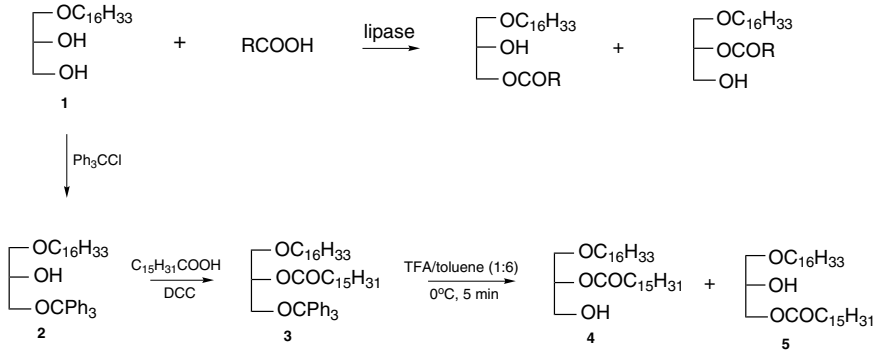
Determination of the optimal temperature. To a solution of 0.05 mmol of 1-*O*-hexadecylglycerol, 0.025 mmol of palmitic acid, and 0.01 mmol of 5-α-cholestane as internal standard in 1 mL of toluene were added 35 units of the lipase from *Pseudomonas* sp., and the mixture was stirred at different temperatures. The reaction progress was monitored by TLC and GLC.

Determination of the optimal solvent. To a solution of 0.05 mmol of 1-*O*-hexadecylglycerol, 0.025 mmol of palmitic acid, and 0.01 mmol of 5-α-cholestane in 1 mL of different solvents were added 35 units of the lipase from *Pseudomonas* sp., and the mixture was stirred at 33°C. The reaction progress was monitored by GLC.

*Chemical synthesis of 1-*O*-hexadecyl-2-*O*-hexadecanoylglycerol (**4**) and 1-*O*-hexadecyl-3-*O*-hexadecanoylglycerol (**5**) (Scheme 1).* A solution of 1-*O*-hexadecylglycerol (**1**) (95 mg, 0.3 mmol), trityl chloride (0.33 mmol), triethylamine (0.45 mmol), and 4-*N*,*N*-dimethylaminopyridine (0.01 mmol) in dichloromethane (2 mL) was stirred overnight at room temperature. The solution was poured into ice water and extracted with dichloromethane. The organic extract was washed with water and dried with sodium sulfate.

After removal of the solvent, compound **2** crystallized from ethanol (yield 161 mg, 96%) and was recrystallized from the same solvent, m.p. 48–49°C, R_f = 0.46 (Solvent ii). The starting material, 1-*O*-hexadecylglycerol, did not move with this solvent. ^{1}H NMR ($CDCl_3$): δ 7.45 (*m*, 6H, aromatic), 7.25 (*m*, 9H, aromatic), 3.95 (*m*, 1H, –C*H*OH–), 3.59–3.41 (*m*, 4H, –CH_2–O–CH_2–), 3.21 (*d*, *J* = 4.7 Hz, 2H, –CH_2OCPh$_3$), 2.42 (*d*, *J* = 4.7 Hz, O*H*, disappears on deuteration). ^{13}C NMR ($CDCl_3$): δ 143.99, 128.77, 127.88, 127.11 (C_6H_5), 86.75 (O*C*Ph$_3$), 72.14, 71.74 (*C*H, O*C*H$_2$R), 69.96 (*C*H$_2$O), 64.75 (*C*H$_2$OCPh$_3$); the alkyl carbons are not listed.

For the acylation, a solution of compound **2** (28 mg, 0.05 mmol), palmitic acid (0.055 mmol), 1,3-dicyclohexylcarbodiimide (0.055 mmol), 4-*N*,*N*-dimethylaminopyridine (0.055 mmol) in 1 mL dichloromethane was allowed to stand overnight at room temperature. The *N*,*N*-dicyclohexylurea was filtered, and the filtrate was washed with water and evaporated to a syrup which was purified by column chromatogra-



SCHEME 1

phy to afford compound **3**, R_f = 0.8 (solvent ii); yield 32 mg (83%). ^{1}H NMR ($CDCl_3$): δ 7.45 (*m*, 6H, aromatic), 7.3 (*m*, 9H, aromatic), 5.22 (*m*, 1H, –C*H*OCOR), 3.61 (*m*, 2H, –CH_2–O–CH_2–), 3.44–3.27 (–O–CH_2–CH_2–), 3.24 (*d*, *J* = 4.7 Hz, 2H, CH_2OCPh$_3$). ^{13}C NMR ($CDCl_3$): δ 143.94, 128.75, 127.82, 127.05 (C_6H_5), 86.75 (O*C*Ph$_3$), 71.73, 71.60 (*C*H, O*C*H_2R), 69.56 (*C*H_2O), 62.73 (*C*H_2OCPh$_3$).

Several methods were tried for detritylation in an attempt to minimize intramolecular acyl migration (12). Best results were obtained by treating tritylated compound **3** with a solution of trifluoroacetic acid (TFA) in toluene (1:6, vol/vol) for 5 min at 0°C. After this time, TLC (solvent i) showed no starting material and two spots, R_f 0.5 (major) and 0.63 (minor) corresponding to the 2-*O* and 3-*O* acyl derivatives, respectively. The latter probably formed in the detritylation step. The reaction was diluted with toluene and evaporated to dryness *in vacuo* at room temperature, with several additions of toluene. The ratio of 1-*O*-hexadecyl-2-*O*-hexadecanoyl-glycerol (**4**) and 1-*O*-hexadecyl-3-*O*-hexadecanoylglycerol (**5**) was established by capillary GLC. The products were separated by column chromatography and characterized by ^{1}H and ^{13}C NMR. For **4**, ^{1}H NMR ($CDCl_3$): δ 5.04–4.95 (*m*, 1H, –C*H*OCO–), 3.81 (*d*, *J* = 4.5 Hz, 2H, CH_2–OCH_2–), 3.65 (*dd*, *J* = 10.6 Hz, *J* = 4.8 Hz, 1H, –CH–CH_2OH), 3.58 (*dd*, *J* = 10.6 Hz, *J* = 5.1 Hz, 1H, –CH–CH_2OH), 3.45 (*t*, *J* = 6.6 Hz, 2H, OCH_2–CH_2), 2.35 (*t*, *J* = 7.35 Hz, 2H, –CH_2–COO–). ^{13}C NMR ($CDCl_3$): δ 173.70 (*C*=O), 72.88 (*C*H), 71.93 (O*C*H_2–R), 70.00 (*C*H_2O), 63.03 (*C*H_2OH). For **5**, ^{1}H NMR ($CDCl_3$): δ 4.21 (*dd*, *J* = 11.5 Hz, *J* = 4.8 Hz, 1H, –CH–CH_2OCO–), 4.12 (*dd*, *J* = 11.5 Hz, *J* = 5.0 Hz, 1H, –CH–CH_2OCO–), 4.07–3.95 (*m*, 1H, –C*H*OH–), 3.55–3.38 (*m*, 4H, –CH_2–OCH_2). ^{13}C NMR ($CDCl_3$): δ 174.00 (*C*=O), 71.80, 71.42 (*C*H, O*C*H_2R), 68.93 (*C*H_2O), 65.44 (*C*H_2OCO).

RESULTS AND DISCUSSION

*Enzymatic esterification of 1-*O*-hexadecyl-*rac*-glycerol.* Esterification of 1-*O*-hexadecyl-*rac*-glycerol with a limited amount of the $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$ fatty acid afforded the monoesters (Scheme 1). Aliquots of the mixtures were analyzed by GLC after trimethylsilylation, using a short capillary column (12 m), under conditions which gave a good separation for the 2-*O*-acyl and 3-*O*-acyl derivatives of each molecular species (Table 1). The absence of the 2,3-di-*O*-acyl derivative was checked by TLC.

The enzymes of *C. cylindracea* or *Pseudomonas* sp. gave the 3-*O*-acyl isomer as the main product together with the 2-*O*-acyl derivative. The products could be separated by Unisil column chromatography, and the alkylglycerol, which was used in excess, could be recovered and recycled. Racemic alkylglycerol was used instead of active compound because of cost. Furthermore, the optically active compounds were not necessary to compare the chromatographic data and characterize the natural acyl derivatives of 1-*O*-alkyl-*sn*-glycerol, for they behave the same under these chromatographic conditions. The positional isomers were identified by comparison with the products obtained by chemical synthesis. The isomers were differentiated by TLC; the 3-*O*-acyl derivative always showed a higher R_f value than the 2-*O*-acyl-1-*O*-alkylglycerol, and the homologous compounds of each type were not separated. In contrast, RPTLC did not differentiate positional isomers but could be used for the separation of the compounds obtained by esterification with homologous fatty acids. Unsaturated derivatives were better characterized by the difference shown by RPTLC before and after catalytic hydrogenation. Chromatographic data are listed in Table 1.

Alkyl monoacylglycerols from the glycoinositol phospholipid membrane anchor of bovine erythrocyte acetylcholinesterase were analyzed as trimethylsilyl ethers by GLC, but no data are given for the retention times of individual species (13).

Effect of the solvent in the lipase synthesis of monohexadecanoyl derivatives of hexadecylglycerol. The esterification of 1-*O*-hexadecylglycerol with palmitic acid was performed at 33°C using the lipase from *Pseudomonas* sp. in toluene, in dichloromethane, and in diisopropylether. The reaction was followed by GLC for several days (Fig. 1). The enzyme was able to esterify the substrate in all three solvents, but was most effective in dichloromethane (yield 75%).

In contrast to our results, esterification of 1-*O*-octadecyl-*rac*-glycerol with oleic acid, catalyzed by porcine pancreatic lipase, does not take place in dichloromethane (9). However, our data agree with those results when using ether as solvent, which extensively inhibited esterification. On the other hand, the enzyme from *C. cylindracea* was able to catalyze the esterification of 2-chloro and 2-methoxy propionic acid with 1-butanol in chloroform (14), and the lipase from *Mucor miehei* was active for the synthesis of propyl myristate in another chlorinated solvent, trichloroethane (15).

As recommended for glycerol esterification (8), the reac-

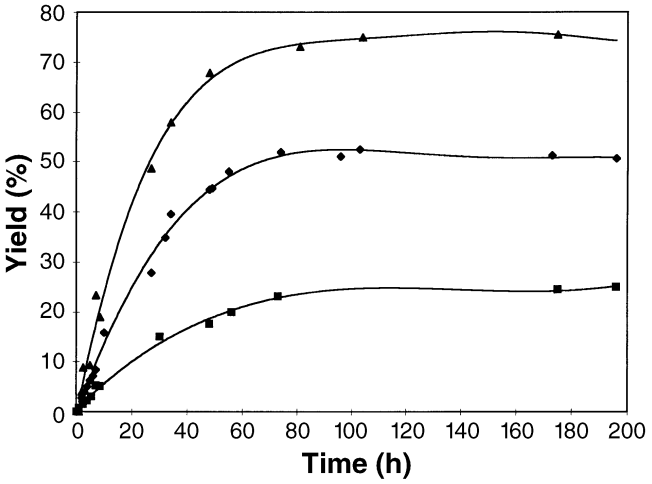


FIG. 1. Effect of solvent in the total yield of monopalmitoyl derivatives of 1-*O*-hexadecylglycerol. Esterification was catalyzed by the lipase from *Pseudomonas* sp. at 33°C. The solvents used were: methylene chloride (▲), toluene (◆), and diisopropylether (■).

tion was also effective in hexane, as shown by TLC, but quantification of the reaction was limited by the low solubility of the substrate. A mixture of the 2- and 3-monoglycerides was obtained in a ratio of 1:4; 1:5, and 1:7 in diisopropylether, toluene, and dichloromethane, respectively, after 72 h, as determined by GLC following trimethylsilylation of an aliquot of the reaction mixture.

Effect of reaction temperature. Figure 2 shows the effect of the reaction temperature on the esterification catalyzed by the *Pseudomonas* lipase in toluene. The rate of the reaction increased with temperature, with a maximum at 55°C. The effect was the same in the other solvents (not shown). In agreement, it was reported that esterification of glycerol with ethyl stearate in acetronile using the lipase from *C. antarctica* increased with temperature, with 60–80°C being the optimal range (16).

*Chemical synthesis of 1-*O*-hexadecyl-2-*O*-hexadecanoyl-rac-glycerol (***4***) and 1-*O*-hexadecyl-3-*O*-hexadecanoyl-rac-glycerol (***5***).* The title compounds were prepared according to Scheme 1, starting from 1-*O*-hexadecyl-*rac*-glycerol. The primary alcohol was selectively protected by tritylation with tritylchloride in triethylamine and 4-*N,N*-dimethylaminopyridine (17). After 18 h no starting material was detected by TLC, and the trityl derivative showed $R_f = 0.46$ (solvent ii). Compound **2** crystallized from ethanol in 96% yield. After recrystallization from the same solvent, it had a m.p. of 48–49°C and was characterized by NMR spectroscopy.

Acylation of **2** was performed with palmitic acid in the presence of 1,3-dicyclohexylcarbodiimide and 4-*N,N*-dimethylaminopyridine, at room temperature (18), affording **3** as an amorphous product with $R_f = 0.80$ (solvent ii) in 83% yield. Enzymatic acylation of compound **2** was not effective with any of the lipases used in this study, probably owing to steric hindrance by the bulky trityl group.

Several methods for the detritylation of compound **3** were tried to minimize acyl migration, a phenomenon well-known in the preparation of 2-monoglycerides (12). A better yield of 1-*O*-hexadecyl-2-*O*-hexadecanoylglycerol (R_f = 0.5, solvent i) was obtained by treating compound **3** with TFA in toluene (1:6, vol/vol) at 0°C. Complete detritylation was obtained after 5 min; however, GLC analysis of an aliquot after trimethylsilylation showed compound **4** and its position isomer **5** in a molar ratio of 2:1. Separation was achieved by column chromatography in a cold room.

Each individual alkylacylglycerol showed a characteristic ^{1}H NMR spectrum for the protons of the glyceryl backbone.

1-*O*-Hexadecyl-2-*O*-hexadecanoyl-*sn*-glycerol has been synthesized by another method, starting from 3-*O*-benzyl-*sn*-glycerol (19). The chemical shifts, which were only reported for position 2 of the glycerol moiety [δ_H 4.99 (*m*) and δ_C 72.91], are similar with those found in the present work for compound **4**.

1-*O*-Hexadecyl-3-*O*-hexadecanoylglycerol has been isolated from a soft coral (20). The NMR data are also in good agreement with our results for compound **5**, although the authors did not assign the ^{13}C NMR signals.

In conclusion, monoesterification of alkylglycerol catalyzed by lipases is a simple method which yields the 2-*O*-acyl and 3-*O*-acyl derivatives in a good total yield. A combination of chromatographic analyses can be used for the identification of the different monoacylalkylglycerols. The 3-*O*-acylglyceride is usually detected together with the natural 2-*O*-acyl derivative as a result of acyl migration.

The monoesters were also obtained by a high-yield chemical method, not previously described for these compounds.

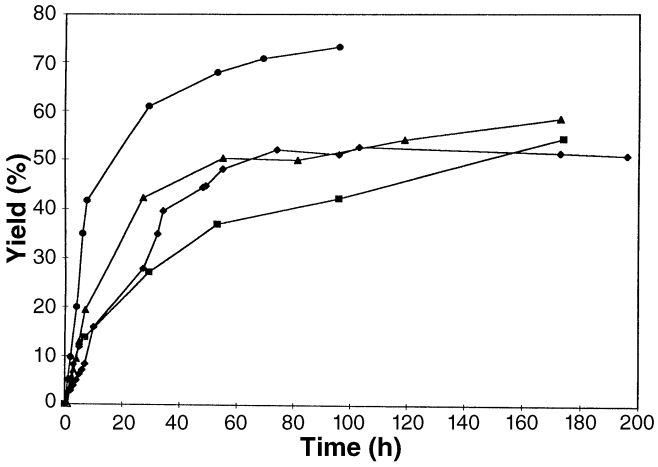


FIG. 2. Effect of reaction temperature in the total yield of monopalmitoyl derivatives of 1-*O*-hexadecylglycerol. Esterification was catalyzed by the lipase from *Pseudomonas* sp. in toluene. The temperatures used were: 25°C (■), 33°C (◆), 45°C (▲), and 55°C (●).

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ARTICLE

Effects of Oxidative Stress on Glycerolipid Acyl Turnover in Rat Hepatocytes

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ABSTRACT: Lipid peroxidation was induced in freshly isolated rat hepatocytes by incubation in the presence of Fe^{3+}, resulting in accumulation of thiobarbituric acid reactive substances. Analysis of lipid classes revealed that the levels and fatty acid compositions of the two major phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), remained unchanged but the levels of triacylglycerols (TAG) were significantly reduced, and some of their polyunsaturated fatty acids were selectively lost as the result of oxidant treatment. Acyl turnover in PC and PE as determined by ^{18}O incorporation from $H_2{}^{18}O$-containing media remained largely unchanged during oxidant treatment, while some increased turnover of the saturated fatty acids in TAG was observed. We hypothesize that constitutive recycling of membrane phospholipids rather than selective *in situ* repair eliminates peroxidized species of PC and PE. TAG could serve as an expendable fatty acid reserve, providing a limited but very dynamic pool of polyunsaturated fatty acids for the resynthesis of phospholipids.
Lipids 32, 917–923 (1997).

Polyunsaturated fatty acids are readily peroxidized by a free radical-driven, autocatalyzed chain reaction (1). In biological systems, lipid peroxidation is kept to a minimum by antioxidants such as tocopherol, ascorbic acid and ubiquinone which break the chain reaction by scavenging free radicals (2,3), and by antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase which inactivate lipid peroxides and reactive oxygen species (4,5). Despite the presence of antioxidant defense systems, a certain extent of peroxidation of the polyunsaturated fatty acids of membrane phospholipids is likely to occur *in vivo*, resulting in the generation of hydroperoxides as primary products. It has been proposed that the combined activities of phospholipase A_2 and acyl-CoA:lysophospholipid acyl transferase could serve as a membrane repair system by selectively removing the peroxidized acyl groups and reacylating the resulting lysophospholipids. Peroxidized fatty acids would be reduced by glutathione peroxidase to hydroxy fatty acids which, in turn, are substrates for degradation *via* β-oxidation (6–9). This hypothesis is based primarily on observations that phospholipase A_2 activity is stimulated by oxidative stress (10–13) and that peroxidized phospholipids are preferentially hydrolyzed by phospholipase A_2 in model membranes (14–16). The proposed reacylation of lysophospholipids that are generated through enhanced phospholipase A_2 activity has received less attention. In order to accomplish repair by this mechanism, enhanced deacylation should be accompanied by enhanced reacylation. However, because phospholipid turnover is a complex process that involves both *de novo* synthesis and remodeling by deacylation–reacylation, oxidative stress may have selective effects on these processes. For example, treatment of endothelial cells with linoleic acid hydroperoxide resulted in enhanced phosphatidylinositol synthesis, as measured by incorporation of both labeled fatty acids and inositol, whereas phosphatidylcholine (PC) synthesis was significantly reduced (17). Also, hydrogen peroxide was found to inhibit reacylation of arachidonic acid into phospholipids of alveolar macrophages (18), and phospholipid reacylation was inhibited in neuronal membranes by lipid hydroperoxides (19). In other radiolabel experiments, enhanced incorporation of exogenous [9,12-^{3}H]oleic acid into phosphatidylethanolamine (PE) was found in *tert*-butylhydroperoxide-treated erythrocytes, whereas its incorporation into PC was inhibited (20).

We have used the incorporation of ^{18}O from $H_2{}^{18}O$-containing media into glycerolipid acyl groups as a measure of acyl turnover in intact cells (21–25). In rat hepatocytes, acyl turnover was high, especially in triacylglycerols (TAG) (20% of total TAG per hour) but also in glycerophospholipids where it involved both *de novo* synthesis and remodeling by deacylation–reacylation at similar rates. We suggested that these two processes are regulated together and are linked to intracellular transfer and degradation (24,25). If all membrane lipids are continually replaced through synthesis, transport and degradation, any peroxidized fatty acids would become substrates for glutathione peroxidase during their normal turnover cycles, thus obviating the need for any specific repair mechanism. However, synthesis and remodeling could be selectively enhanced under oxidative stress.

In the present study, we have used the ^{18}O-technique to determine the effects of oxidative stress on glycerolipid acyl turnover in rat hepatocytes. We have found that acyl turnover

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Abbreviations: NEFA, nonesterified fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol(s); TBARS, thiobarbituric acid-reactive substances.

remains essentially unchanged during peroxidative treatment and that TAG are selectively depleted of their polyunsaturated fatty acids. Our results suggest that membrane repair occurs by a process based on recycling rather than on selective deacylation–reacylation of peroxidized phospholipids *in situ*.

MATERIALS AND METHODS

Materials. Male Sprague-Dawley rats, 200–350 g, were obtained from Sasco, Inc. (Omaha, NE). $H_2{}^{18}O$, 95% enrichment, was from Isotec, Inc. (Miamisburg, OH). Tetramethoxypropane, diheptadecanoylPC, diheptadecanoylPE, and $FeCl_3$ were from Sigma (St. Louis, MO). Heptadecanoic acid and triheptadecanoylglycerol were from Nu-Chek-Prep (Elysian, MN). Gas chromatography–mass spectrometry was carried out on a Hewlett-Packard 5890 series gas chromatograph (Palo Alto, CA) with a 5972 mass-selective detector and automatic injection system.

Hepatocyte preparation and incubation. Hepatocytes were obtained by perfusion of the liver with collagenase (26) using nonfasted rats as previously described (24). Viability was assessed by determination of trypan blue exclusion (24) and/or lactate dehydrogenase leakage (27), and was always found to be above 85%. Hepatocytes were resuspended in Krebs-Ringer/HEPES buffer pH 7.4 (1.5 mM Ca^{2+}) with 10 mM glucose and no bovine serum albumin at a density of 4×10^6 cells/mL. Cells were incubated for up to 90 min in 15-mm diameter glass vials (0.2 mL incubates) at 37°C with gentle swirling in an atmosphere of 95:5, O_2/CO_2. For experiments of ^{18}O incorporation, the incubation medium contained 40% $H_2{}^{18}O$. In the cases in which lipid peroxidation was induced, $FeCl_3$ (to a final concentration of 93 μM) was added from a freshly prepared concentrated solution at the start of incubations. Thiobarbituric acid reactive substances (TBARS) were determined in aliquots of total incubation media according to Huber *et al.* (28), and were expressed as nmol of malondialdehyde equivalents, using tetramethoxypropane as standard. In this procedure, boiling of samples is avoided in order to prevent interference of products derived from sugars (28). After incubation, the cell suspensions were transferred to ice-cold glass tubes, and the cells were sedimented by centrifugation at low speed. Lipids were extracted immediately from the cell pellets (29) using solvents containing 0.01% butylated hydroxytoluene. Lipid phosphorus was determined in aliquots according to Bartlett (30).

Separation of lipid classes and preparation of fatty acid methyl esters. Lipid classes were obtained by preparative thin-layer chromatography on layers of Silica Gel H, 20 × 20 cm. Plates were viewed under ultraviolet light after spraying with 2′,7′-dichlorofluorescein (0.1% wt/vol in ethanol), and fractions were scraped off. For preparation of PC and PE chromatograms were developed with chloroform/methanol/ammonium hydroxide/water (65:35:5:1, by vol), and the phospholipids were eluted from the adsorbent with chloroform/methanol/water (30:50:20, by vol). Nonesterified fatty acids (NEFA) methyl esters and TAG were obtained by thin-layer chromatography of aliquots in which NEFA had been methylated with diazomethane (31). Hexane/diethyl ether/glacial acetic acid (80:20:1, by vol) was used as developing system and diethyl ether for elution. Methyl esters of esterified fatty acids in PC, PE, and TAG were prepared by transesterification in 0.2 N NaOH/methanol (45°C, 1 h).

Fatty acid composition and ^{18}O excess. Fatty acid methyl esters were analyzed, and ^{18}O excess was determined by gas chromatography–mass spectrometry using a Hewlett-Packard HP5MS 30 m × 0.25 mm column. DiheptadecanoylPE, diheptadecanoylPC, triheptadecanoylglycerol, and heptadecanoic acid were added to the original lipid extracts as internal standards. The methyl ester preparations were divided into two aliquots. One of them was hydrogenated and used for determination of ^{18}O excess in the resulting 16:0, 18:0, 20:0, and 22:0 methyl esters by gas chromatography–mass spectrometry as described (22–24), using the McLafferty rearrangement ion (*m/z* 74 and *m/z* 76). Unlabeled hepatocyte preparations were processed and used to correct for the natural abundance of *m/z* 76 (about 1% of *m/z* 74). The second aliquot was not hydrogenated and was used for determination of ^{18}O excess in palmitic (16:0) and stearic (18:0) acid methyl esters. Catalytic hydrogenation of unsaturated fatty acids is required to achieve accurate measurement of ^{18}O excess (22–24). Thus, ^{18}O excess is given on figures and tables for the methyl esters of palmitic and stearic acids analyzed in nonhydrogenated samples, and for the methyl esters of C_{20} and C_{22} fatty acids analyzed in hydrogenated samples, representing mostly arachidonic (20:4) and docosapentaenoic (22:5) plus docosahexaenoic (22:6) acids, respectively. Additionally, ^{18}O excess in the methyl esters of oleic plus linoleic acids was obtained by calculating the difference between the values for the methyl ester of stearic acid (18:0) in hydrogenated and nonhydrogenated samples. This second, nonhydrogenated aliquot also was used for determination of fatty acid composition using total ion detection. All experiments were carried out in duplicate and were well reproducible. In all figures, high and low values are superimposed on the bar graphs representing averages.

RESULTS

Effects of oxidative stress on lipid class and fatty acid composition. Lipid peroxidation was induced in isolated rat hepatocytes by incubation in the presence of 93 μM Fe^{3+}. This led to accumulation of TBARS, which include malondialdehyde and other secondary products of lipid peroxidation (32) (Fig. 1). The lipid-soluble antioxidant, butylated hydroxytoluene completely inhibited TBARS generation caused by Fe^{3+}. Although TBARS accumulated faster during the first 20 min of oxidant treatment, accumulation continued throughout the 90-min incubation, indicating that lipid peroxidative decomposition went on during the entire time period. Viability of control and oxidant-treated cells remained relatively high, declining by about 8% from 85–90% at the beginning of the incubations. Hence, assays were performed on cells incubated in the presence or absence of Fe^{3+} for 45 and 90 min.

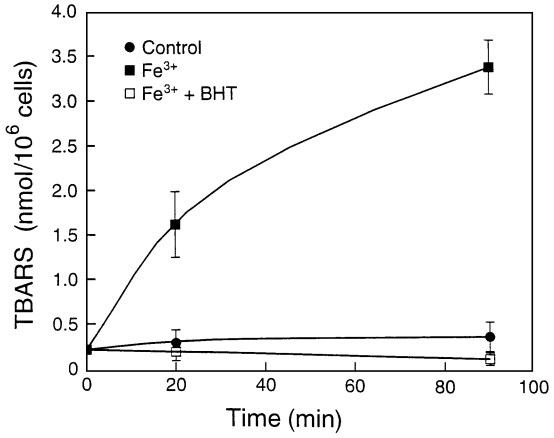

FIG. 1. Thiobarbituric acid-reactive substances (TBARS) accumulation during incubation of hepatocytes with no addition, addition of 93 μM Fe^{3+}, and addition of 93 μM Fe^{3+} plus 2 mM butylated hydroxytoluene (BHT). Cells were preincubated for 5 min with or without BHT before addition of Fe^{3+}. TBARS were determined in 50 μL aliquots taken from the incubations as described in the Materials and Methods section. Averages and standard deviation of three different incubations are shown.

In spite of the detectable lipid peroxidation (TBARS accumulation) as the result of treatment with Fe^{3+} (Fig. 1), the cells did not exhibit any measurable loss of polyunsaturated fatty acids (Table 1).

When the major classes of glycerolipids were isolated and analyzed separately, the results summarized in Table 2 were obtained. Oxidant treatment had little or no effect on the levels of PC and PE, based on total lipid phosphorus, but caused a significant reduction of TAG. Both control and oxidant-treated cells showed a decline in total phospholipid of 5 and 10% as determined by phosphorus analysis of aliquots after 45 and 90 min of incubation, respectively. This was probably as a result of a decrease in intact cell numbers and/or losses of membranes due to blebbing (33). As illustrated in Figure 2, there were only minor changes in the constituent fatty acids of PC and PE, but there was a selective loss of polyunsaturated fatty acids in TAG after 90 min of oxidant treatment.

TABLE 1
Fatty Acid Composition (mol %) of Rat Hepatocyte Total Lipids Before and After Treatment with 93 μM Fe^{3+}[a]

	No Incubation	Incubation			
		45 min		90 min	
		Control	Fe^{3+}	Control	Fe^{3+}
16:0	20.2	20.0	20.4	21.6	20.2
18:0	20.7	22.2	21.3	22.7	23.1
18:1	12.6	15.1	12.9	13.9	14.1
18:2n-6	18.3	15.2	18.2	15.7	14.9
20:4n-6	18.9	19.2	19.1	17.9	19.0
22:5n-3	1.7	1.5	1.7	1.6	1.5
22:6n-3	7.7	6.8	6.4	6.6	7.2

[a]Fatty acid methyl esters were prepared by transesterification in 0.2 N NaOH/MeOH and analyzed by gas chromatography using 17:0 as internal standard. Minor fatty acids, together representing less than 5% of the total (16:1, 20:3n-6, and 20:5n-3), were not routinely determined. Data represent averages of duplicate incubations (see figures for typical variability of data).

As shown in Figure 3, oxidant treatment for 90 min also reduced the levels of NEFA by 60 to 80%, whereas 45 min of oxidant treatment had essentially no effect. As observed previously (24), rat hepatocytes contain very low levels of NEFA (9 nmol/μmol lipid phosphorus), consisting almost entirely of palmitic, stearic and oleic acids, and they do not contain measurable amounts of polyunsaturated fatty acids.

Effects of oxidative stress on glycerolipid acyl turnover. Incorporation of ^{18}O from $H_2{}^{18}O$-containing media into glycerolipid acyl groups was used as a measure of acyl turnover. Control cells exhibited acyl turnover similar to that observed previously under the same conditions (24,25). ^{18}O incorporation into all acyl groups increased linearly during incubation except for a small decline in cells incubated for 90 min with Fe^{3+} (Fig. 4). In PC (Fig. 5), ^{18}O-incorporation was linear for

TABLE 2
Fatty Acid (FA) Composition of the Major Classes of Glycerolipids in Isolated Hepatocytes[a]

	Phosphatidylcholine					Phosphatidylethanolamine					Triacylglycerols				
	0 min	45 min		90 min		0 min	45 min		90 min		0 min	45 min		90 min	
		Control	Fe^{3+}	Control	Fe^{3+}		Control	Fe^{3+}	Control	Fe^{3+}		Control	Fe^{3+}	Control	Fe^{3+}
FA (mol %)															
16:0	26.9	24.6	24.0	24.9	24.9	24.7	24.4	25.1	25.2	24.8	37.1	36.8	36.1	33.6	37.1
18:0	26.0	24.3	23.3	25.6	24.3	31.0	30.6	31.0	30.1	30.9	8.6	10.0	9.2	8.7	9.0
18:1	4.4	4.4	4.5	4.4	4.7	3.1	3.0	2.8	2.8	2.7	27.4	27.5	27.1	26.9	27.6
18:2n-6	22.7	23.3	23.4	21.7	22.9	11.4	10.7	11.2	11.4	12.1	18.5	18.2	20.4	20.8	19.2
20:4n-6	14.2	16.6	17.1	16.6	16.3	18.2	18.5	18.2	18.1	17.9	2.5	2.5	2.6	3.5	2.6
22:5n-3	1.4	1.3	1.2	1.0	1.1	2.0	2.2	2.0	2.0	2.0	1.6	1.5	1.5	2.0	1.3
22:6n-3	5.1	6.2	6.5	5.8	5.9	9.5	10.2	9.6	9.6	9.6	3.3	3.4	3.2	4.5	3.0
nmol FA/μmol total LP	787.9	849.0	866.6	876.5	841.4	386.0	412.0	385.5	420.5	409.4	388.4	369.4	319.4	381.8	296.9
nmol lipid class/μmol total LP	393.5	424.5	433.3	438.2	420.7	193.0	206.0	192.7	210.2	204.7	129.5	123.1	106.5	127.3	99.0

[a]Before and after incubation for 45 and 90 min in the absence or presence of 93 μM Fe^{3+}. Lipid classes were separated by preparative thin-layer chromatography and fatty acid methyl esters prepared as described in the Materials and Methods section. 17:0 Internal standards for each lipid class were used. Averages of duplicate incubations are shown. LP: lipid phosphorus.

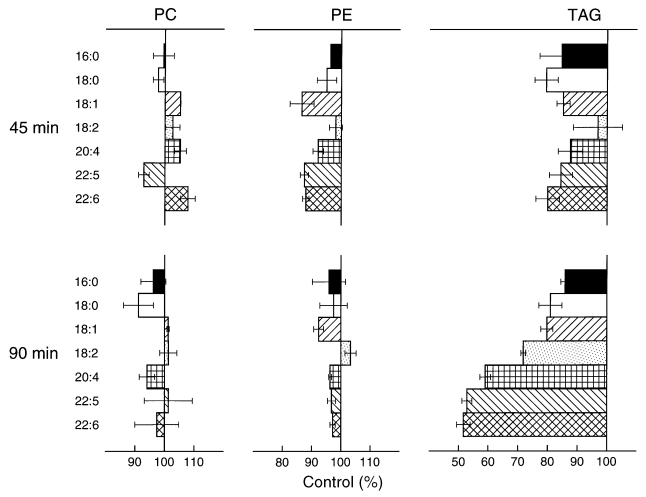

FIG. 2. Effect of Fe^{3+} treatment on the fatty acid composition of the major lipid classes. Fatty acid compositions were determined in phosphatidylcholine (PC), phosphatidylethanolanine (PE), and triacylglycerols (TAG) of hepatocytes incubated for 45 and 90 min in the presence or absence of 93 μM Fe^{3+}. Fatty acids were calculated as nmol of fatty acid per mol of total lipid phosphorus (Table 2), and their composition in the peroxidized hepatocytes was then expressed as percentage of their composition in control cells incubated for the same time periods. Averages of duplicate incubations are represented. High and low values are superimposed.

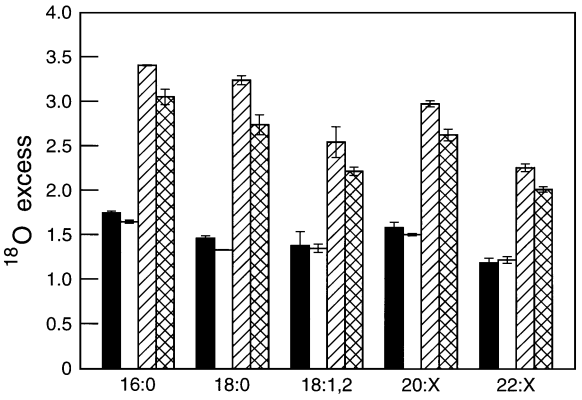

FIG. 4. Incorporation of ^{18}O from $H_2{}^{18}O$ into hepatocyte total fatty acids. Lipids were extracted from hepatocytes incubated for 45 and 90 min in media containing 40% $H_2{}^{18}O$ in the presence and absence of 93 μM Fe^{3+}. Atom percentage excess of ^{18}O was determined by gas chromatography–mass spectroscopy of fatty acid methyl esters as described in the Materials and Methods section. Averages of duplicate incubations are represented, including high and low values. Closed box, 45-min control; open box, 45-min Fe^{3+}; diagonal striped box, 90 min control; cross-hatched box, 90 min Fe3+.

90 min and proceeded at 1.3 atom percentage excess per hour for 16:0 and 18:0, 1.1% for 18:1 plus 18:2, 1.75% for 20:4, and 0.4% for the C_{22} fatty acids. The data for PE were similar except that incorporation was lower in 20:4 (0.8%) but higher in the C_{22} fatty acids (1.1%). Perhaps most importantly, oxidative stress for 45 and 90 min had no measurable effect on the rates of acyl turnover of these two major classes of glycerophospholipids. As observed previously (24), acyl turnover was higher in TAG than in either PC or PE, especially with respect to the long-chain polyunsaturated C_{20} and C_{22} fatty acids of which arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) are the major representatives. Oxidant treatment resulted in increased turnover of 16:0 and 18:0 in TAG but had no effect on ^{18}O incorporation into NEFA (data not shown).

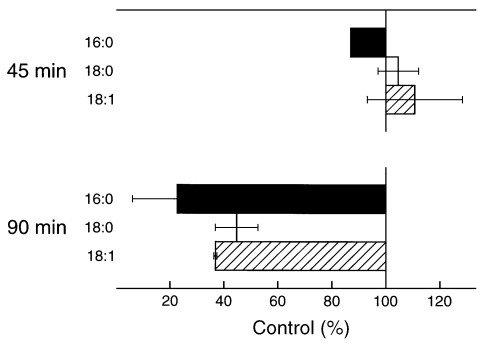

FIG. 3. Effect of Fe^{3+} treatment on nonesterified fatty acids. Methyl esters of nonesterified fatty acids were obtained by reaction with diazomethane and isolated by preparative thin-layer chromatography from lipid extracts of hepatocytes incubated for 45 and 90 min in the presence and absence of 93 μM Fe^{3+}. In using heptadecanoic acid as internal standard, fatty acid composition was calculated as nmol of fatty acid per mol of total lipid phosphorus, and the values for peroxidized hepatocytes were then expressed as percentage of those for control cells incubated for same time controls. Averages of duplicate incubations are represented, including high and low values.

Table 3 summarizes ^{18}O incorporation (calculated from data of the 45-min incubations) for the three major classes of glycerolipids. By taking into account the concentration of $H_2{}^{18}O$ in the media (40%) and the loss of one fatty acid oxygen upon reacylation, acyl turnover rates can be estimated from atom percentage excess of ^{18}O by multiplying these data by a factor of 5 (22–24). Thus, acyl turnover in hepatocyte PC and PE proceeded at up to 10% per hour. Perhaps most importantly, turnover of polyunsaturated fatty acids was extremely high in TAG, i.e., ~40% per hour for C_{20} and ~20% per hour for C_{22} fatty acids (Table 3). Even though these fatty acids are relatively minor components of TAG (Table 2), they are continuously recycled and may therefore become readily available for phospholipid synthesis and remodeling.

DISCUSSION

We have induced oxidative stress in isolated rat hepatocytes by incubating them in the presence of Fe^{3+}. Iron propagates lipid peroxidation by catalyzing the decomposition of preexisting lipid hydroperoxides to form alkyl and peroxyl radicals (34), and also may play a role as initiator of lipid peroxidation (35). The use of Fe^{3+} to induce lipid peroxidation in hepatocytes is well documented, especially when used as the Fe^{3+}/ADP complex (36–38). However, we observed that ADP

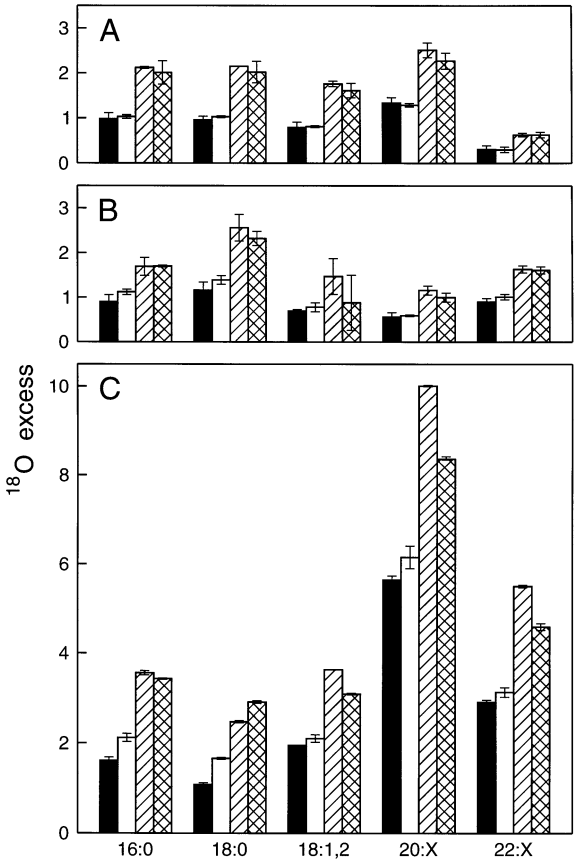

FIG. 5. Incorporation of ^{18}O from $H_2{}^{18}O$ into constituent fatty acids of the major lipid classes. Lipids were extracted from hepatocytes incubated for 45 and 90 min in media containing 40% $H_2{}^{18}O$, in the presence and absence of 93 μM Fe^{3+}. PC (panel A), PE (panel B), and TAG (panel C) were isolated by preparative thin-layer chromatography and ^{18}O excess was determined by gas chromatography–mass spectrometry of fatty acid methyl esters as described in the Materials and Methods section. Averages of duplicate incubations are represented, including high and low values. Closed box, 45 min control; open box, 45 min Fe^{3+}; diagonal striped box, 90 min control; cross-hatched box, 90 min Fe^{3+}.

itself caused important lipid metabolic alterations, independent of oxidative stress (39). Hence, we have used Fe^{3+} alone which caused nonlethal lipid peroxidation as indicated by the accumulation of TBARS (Fig. 1). This oxidative stress did not affect the amounts, acyl compositions, and acyl turnover rates of the major glycerophospholipids, PC and PE. In contrast, the amounts of TAG were reduced, concomitant with a selective loss of their polyunsaturated fatty acids. Assuming that lipid peroxidation affects both phospholipids and TAG, we propose that the selective losses of polyunsaturated fatty acids from TAG, which might be considered an expendable fatty acid pool, are used to maintain the structurally and functionally important glycerophospholipids. Lipolytic release of arachidonate from TAG and its incorporation into glycerophospholipids have been demonstrated in cultured human leukemia (HL-60) cells (40). Pacifici *et al.* (17) speculated that TAG could sustain membrane turnover in endothelial cells treated with a lipid hydroperoxide by providing diacylglycerol for phospholipid synthesis. Utilization of polyunsaturated fatty acid from the TAG pools could satisfy immediate, short-term needs, and TAG is the only source of such fatty acids in cultured cells. Supply of polyunsaturated fatty acid to cells *in vivo* occurs through the blood supply.

The membrane phospholipids of hepatocytes and other nucleated cells exist in a dynamic equilibrium involving *de novo* synthesis, remodeling by deacylation–reacylation, intracellular transfer, and degradation. We have shown that this process involves the turnover of substantial portions (>10% per hour) of the major glycerophospholipids (21–25). Hence, peroxidatively modified acyl groups would be hydrolyzed readily during normal glycerolipid turnover. Our present data support the view that this constitutive acyl turnover of glycerophospholipids has a major role in protecting cell membranes from lipid peroxidation. A preferential hydrolysis of peroxidized acyl groups by phospholipase A_2 (14–16) could be part of this process by making it more likely for peroxidized phospholipids to be recycled, without causing measurable increases in the overall rates of turnover. The lysophospholipids generated by this mechanism could be removed by lysophospholipase activity rather than being substrates for enhanced reacylation. Lysophospholipases are widely distributed in rat liver and other tissues, exhibit broad substrate specificity, and are thus able to prevent the accumulation of lysophospholipids (41). We have not found any evidence of the proposed *in situ* repair or of a nonspecific, general increase of acyl turnover, which also might be a possible response to peroxidative stress. It will be of interest to examine the effects of oxidant

TABLE 3
Incorporation of ^{18}O from $H_2{}^{18}O$ into the Glycerolipids of Hepatocytes Incubated in the Presence and Absence of 93 μM Fe3+[a]

	Phosphatidylcholine				Phosphatidylethanolanine				Triacylglycerols			
	Control		Fe^{3+}		Control		Fe^{3+}		Control		Fe^{3+}	
	A	B	A	B	A	B	A	B	A	B	A	B
16:0	1.30	6.5	1.37	6.9	1.19	6.0	1.49	7.5	2.15	10.8	2.83	14.2
18:0	1.28	6.4	1.37	6.9	1.54	7.7	1.85	9.3	1.44	7.2	2.21	11.1
18:1 + 18:2	1.05	5.3	1.08	5.4	0.91	4.6	1.04	5.2	2.60	13.0	2.80	14.0
20:4	1.79	9.0	1.73	8.7	0.74	3.7	0.78	3.9	7.51	37.6	8.20	41.0
22:5 + 22:6	0.41	2.1	0.40	2.0	1.20	6.0	1.35	6.8	3.87	19.4	4.17	20.9

[a]A: ^{18}O content (atom % excess per h); B: acyl turnover (percentage per hour). Data represent averages of two separate incubations. Turnover rates were calculated by multiplying atom percentage excess by a factor of 5.

treatment on glycerolipid acyl composition and turnover in mammalian cells other than hepatocytes which are specialized for *de novo* glycerolipid synthesis and lipoprotein assembly.

ACKNOWLEDGMENTS

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The Effects of Cyclic Fatty Acid Monomers on Cultured Porcine Endothelial Cells

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ABSTRACT: The popularity of polyunsaturated oils used in food applications and preparation continues to appreciate as a result of positive health claims. With polyunsaturated oils inherently more susceptible to oxidative and thermal degradation, the formation of new fatty acid species increases considerably. The presence of one species known as cyclic fatty acid monomers (CFAM) has been detected as a component of many oils subjected to various thermal processes including deep-fat frying. The effect of CFAM on metabolic processes has not been fully characterized. In this study, confluent porcine aortic endothelial cells incorporated CFAM into their polar and nonpolar lipid fractions following a 48-h exposure to 31 and 62 ppm CFAM in the culture medium. Subsequently, the influence of CFAM incorporation on various membrane-dependent physical properties and biochemical processes was investigated. CFAM decreased the lipid packing order of the membrane bilayer core but did not alter the lipid packing order of lipid chain segments at or near the lipid–water interface of the membrane. CFAM led to significant reductions in Ca^{2+} ATPase activity and monolayer integrity while eliciting a significant increase of prostacyclin synthesis and secretion.

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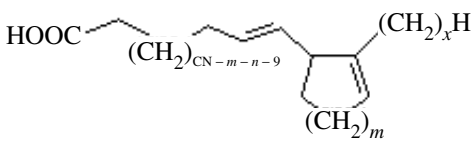
During the past two decades, a great deal of progress has been made toward defining the role of dietary fats in the pathogenesis of certain Western diseases. For numerous such conditions including cardiovascular disease and certain cancers, the type and amount of dietary fat have been implicated as positive risk factors (1–4). Several basic pathogenic mechanisms associated with these diseases often can be characterized by altered cell function including aberrant biochemical processes resulting from physical changes at the phospholipid membrane level (5,6). The influence of dietary lipids becomes significant in membrane-dependent properties owing to the importance of fatty acids in membrane phospholipid structure.

In particular, the pathogenesis of vascular disease suggests that dietary fatty acids play an important role especially owing to their influence on the function of the vascular endothelium. Incorporation of polyunsaturated fatty acids (PUFA) into cultured endothelial cells has been shown recently to promote disruption of normal endothelial cell functions (7–9) which would appear to contradict the current hypothesis that PUFA minimize the incidence of atherosclerotic episodes. Furthermore, the inherent instability of PUFA to oxidative and thermal stress can lead to the formation of a host of potential lipid degradation and oxidation products, caused by *in vivo* processes and/or dietary consumption, which subsequently may be presented to endothelial cells .

A popular source of ingested lipid breakdown products is the consumption of deep-fat fried foods. In the case of vegetable oils used in deep-fat frying processes, the formation of lipid oxidation products can be substantial even though a majority of vegetable oils are modified for increased stability by reducing the degree of unsaturation using catalytic hydrogenation. The formation of these partially degraded lipid products can be attributed to the exposure of the frying medium to water (from food), oxygen, metals (from food or equipment), and the high temperature of the frying process (10,11). Various lipid breakdown products have been identified in frying fats and oils derived from both animal and vegetable sources (12,13) and are absorbed by the fried food (14–16). If a frying medium has been abused and overused, accumulation of lipid breakdown products can be significant (17,18).

Cyclic fatty acid monomers (CFAM) are a class of lipid oxidation products generated especially in heated vegetable oils (19–21). A ring structure is formed within the same fatty acyl chain to create a CFAM molecule [Scheme 1 where *x*



SCHEME 1

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Abbreviations: BSA, bovine serum albumin; CFAM, cyclic fatty acid monomer; DPH-PA, 1,6-diphenyl-1,3,5-hexatriene propionic acid; FAME, fatty acid methyl esters; FBS, fetal bovine serum; PUFA, polyunsaturated fatty acids; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene.

represents number of methylene groups determined by the formula: CN (carbon number) − *m* − *n* − 9] as a result of a new covalent linkage between two nonadjacent carbon atoms. Because of their unique structure, the physical characterization of CFAM has been under investigation since their identification in heated oils and only recently have novel analytical techniques been applied to definitively confirm the structure of CFAM (22–24).

The metabolic and physiologic effects of fatty acids and lipid oxidation products in tissue culture systems have been reported in the literature (25–29). While CFAM from heated linseed oil have specific effects on the electrophysiological function of cultured rat myocardiocytes (30), the focus of this study was to examine the influence of CFAM derived from heated linseed oil on various physical and biochemical cellular parameters of cultured endothelial cells using measurements of membrane lipid packing order, membrane integrity/permeability, prostacyclin synthesis, and ATPase activity.

EXPERIMENTAL PROCEDURES

Preparation and isolation of CFAM. Linseed oil (Cargill, Riverside, ND) was heated at 275°C under nitrogen for 12 h as described previously (21). The protocol for the isolation and purification of CFAM includes saponification of the heated linseed oil, esterification of fatty acids, separation of fatty acid methyl esters (FAME) from polar compounds using silicic acid column chromatography, and isolation of CFAM methyl esters by urea fractionation of the nonpolar FAME fraction (21,31). The urea fractionation step was conducted twice using a 3:1 ratio (w/w) of urea to FAME.

Preparation of primary endothelial cell culture. Endothelial cells were isolated from a 6- to 8-inch section of the descending aortas of freshly slaughtered 5- to 8-mon-old pigs. Two- to three-inch segments of aortas were meticulously trimmed of fat and serosa, branch points ligated, rinsed with modified Hank's buffered salts solution, and then filled with 0.1% collagenase solution (ICN Biomedicals, Costa Mesa, CA). Following incubation for 30 min at 37°C, the enzyme/cell solution was decanted then centrifuged at 200 × *g* for 5 min. The pellet was resuspended in Medium 199 (M-199) containing 10% fetal bovine serum, 100 μg/mL heparin, and 100 μg/mL streptomycin. The cell suspension was plated on 60-mm culture dishes and incubated at 37°C in a humidified 5% CO_2 atmosphere.

After 12 h, the medium was changed to remove any nonadherent material, and the adherent cells were viewed under an inverted microscope equipped with Hoffman modulation. The cells had a cobblestone morphology typical of cultured endothelial cells. The cells were allowed to grow to confluence then were passaged at a dilution factor of 1:3. Further characterization of the endothelial monolayer was performed using factor VIII histochemical staining [1° antibody-goat antihuman factor VIII (ICN Biomedicals); 2° antibody-rabbit antigoat labeled with fluoroscein isothiocyanate (Cappel Labs, Cochransville, PA)], and diI-acetylated low density lipoprotein (Biomedical Technologies Inc., Boston, MA). The fluorescence staining techniques revealed the cultures to be essentially pure endothelial cells. Beginning at passage 4, cells were frozen in the M-199 growth medium containing 10% dimethylsulfoxide; then they were stored in liquid nitrogen until use.

Treatment of endothelial cells with medium containing CFAM. The culture medium containing CFAM was prepared by adding the sodium soaps of CFAM to M-199 containing fatty acid free bovine serum albumin (BSA) (Fraction V; ICN Biomedicals) at a molar ratio of 6:1, respectively. The fatty acid-free BSA was slowly dissolved in M-199 at 37°C under constant stirring. The sodium soaps were prepared by the addition of 6 N NaOH to CFAM (free fatty acid form) dissolved in hexane. The solution was dried under nitrogen then redissolved in warm water. The sodium soaps were added dropwise followed by mild stirring for 3–4 h at 37°C. The CFAM–BSA solution was sterilized by passage through a 0.2-μm cellulose acetate filter under vacuum. This solution was used as stock for dilution with M-199 to the desired levels of CFAM in the medium. Two concentrations of CFAM at 31 and 62 parts per million (ppm) were selected which corresponded to 112 and 224 μM, respectively. These concentrations fall within the spectrum of fatty acid supplementation levels in cell culture systems previously described in the literature (7,8,38,40,41).

Isolation of total cellular lipids and separation of nonpolar and polar fractions. Following incubation with CFAM for 48 h, a total lipid extract was isolated from freshly homogenized endothelial cells using the method described by Folch *et al.* (32) and stored at −80°C until further use. The total cellular lipid extract was separated into nonpolar and polar fractions using silica gel solid phase extraction (33). The extraction was confirmed by silica gel thin-layer chromatography using a mobile phase of hexane/diethyl ether/methanol/acetic acid (90:20:5:2, by vol) and visualized by the application of concentrated sulfuric acid/water (1:1, vol/vol) containing 0.05% potassium dichromate followed by heating at 120°C for 10–15 min.

Determination of phospholipid classes using high-performance liquid chromatography. The relative percentage of the major phospholipid classes present in the polar lipid fraction was determined by high-performance liquid chromatography using the method developed by Christie (34). The separation of phospholipid classes was achieved using a modified tertiary gradient mobile phase system with a Spherisorb silica-3 column (Alltech, Deerfield, IL), and the separated phospholipid components were detected using an evaporative light-scattering detector (Varex, Rockville, MD).

Determination of cholesterol and fatty acid composition using gas–liquid chromatography (GLC). The cholesterol content in the nonpolar lipid fraction was quantitated by GLC analysis using stigmasterol as an internal standard wtih an HP-5 column (Hewlett-Packard, Avondale, PA) installed in an HP 5890 Series II Plus GC (Hewlett-Packard).

The fatty acid composition of the nonpolar and polar lipid fractions as well as M-199 containing 5% fetal calf serum was determined by GLC of native and completely hydrogenated FAME (35). FAME mixtures were subjected to GLC analysis using a CP-Sil 88 column (Chromapack, Raritan, NJ) installed in an HP 5890 Series II GC (Hewlett-Packard).

Hydrogenation. CFAM methyl esters and cellular FAME were catalytically hydrogenated over platinum oxide using a microhydrogenator (Supelco, Inc., Bellefonte, PA) (20). Briefly, 100–150 mg CFAM was added to a thick-walled glass reaction tube, then diluted in 20 mL anhydrous methanol. Platinum oxide (10–15 mg) was added under constant stirring. The reaction chamber was evacuated then pressurized with 10 psi hydrogen. After 30 min, the reaction mixture was filtered through a 25-mm filter disk containing a polytetrafluoroethylene membrane with a pore size of 0.2 μm. The methanol was evaporated in a warm water bath under a stream of nitrogen.

Measurement of CFAM cytotoxicity. Endothelial cells were grown to confluence then exposed to CFAM-containing media for 48 h. The cell number was determined using a hemocytometer, and viablility was assessed by trypan blue dye exclusion.

Measurement of membrane lipid packing order. The measurement of membrane lipid packing order of cultured endothelial cells was accomplished using methods adapted from Block *et al*. (36) and Sheridan and Block (37). Dislodged endothelial cells (1×10^6 cells) were suspended in M-199 with 10% FBS, centrifuged, decanted, diluted in 0.3 mL of ice-cold 50 mM phosphate buffer containing 0.15 M potassium chloride at pH 7.4 (phosphate buffer), and stored on ice until preparation for the membrane polarization determination. The cell suspension was warmed to room temperature and an equal volume of 10 μM 1,6-diphenyl-1,3,5-hexatriene propionic acid (DPH–PA) or 20 μM DPH working solutions in phosphate buffer was added to the cell suspension. Following mixing, the cell-fluorescence probe reaction mixtures were allowed to incubate in the dark for 20 min at room temperature. The cell–probe mixture was centrifuged and rediluted in 1 mL phosphate buffer warmed to 37°C. The cell suspension was placed in a quartz cuvette containing 1 mL of phosphate buffer at 37°C. The anisotropy was determined at 37°C using an ISS spectrofluorometer (ISS, Bellefonte, PA) configured in the L-format. The excitation and emission wavelengths were 366 and 430 nm, respectively, for DPH–PA, 365 and 428 nm, respectively, for 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA–DPH), and 362 and 430 nm, respectively, for DPH. A cell suspension prepared without the fluorescence probes was used to correct for background fluorescence. The use of cutoff filters was employed to minimize the effects of light scattering.

Measurement of monolayer integrity. Monolayer integrity was assessed by measuring albumin transfer across a confluent endothelial cell monolayer as described by Henning *et al*. (38). Endothelial cells were seeded at confluence density (1.25×10^5 cells/cm^2) on Cellagen 24-well plate inserts (ICN Biomedicals). The cells were incubated for 8 h to ensure that the cells adhered to the Cellagen inserts then observed for monolayer confluence by microscopic examination. The cells were treated with CFAM media for 48 h. Following the removal of media from the insert (upper) and well (lower) chambers, the concentration of BSA in the media from the lower chamber was detected by measuring the absorbance at 630 nm following reaction with bromocresol green.

Measurement of calcium ATPase activity. Calcium ATPase activity in cultured endothelial cells was assayed using the procedure of Henning *et al*. (8). Briefly, dislodged endothelial cells ($2–3 \times 10^6$ cells) were suspended in M-199 with 10% FBS, centrifuged, decanted, and diluted with 0.25 mL of ice-cold 0.1 M Tris buffer at pH 7.4 and placed on ice. The cell suspension was subjected to ultrasonic homogenization for 15 s in an ice bath. Calcium ATPase activity was assayed following the addition of 50 μL cell homogenate to the following reaction mixture: 500 μL of 0.1 M Tris buffer, pH 7.4; 50 μL of 0.1 M $CaCl_2$; 50 μL of 0.01M ATP. The sample was incubated for 15 min at 37°C, and the reaction was stopped by adding 0.5 mL of cold trichloroacetic acid and the inorganic phosphorus content was determined (39). Total protein was determined by a protein assay kit (Pierce, Rockford, IL).

Measurement of prostacyclin synthesis. Confluent monolayers of endothelial cells in 24-well plates were treated with medium containing CFAM–BSA or BSA alone. Indomethacin (5 μM), a cyclooxygenase inhibitor, was employed to inhibit prostacyclin synthesis, and this incubation served as a positive control. At various times, the medium was removed and was frozen at −20°C until analysis for prostacyclin. The prostacyclin content was determined by measuring the amount of 6-keto-$PGF_{1\alpha}$, its stable metabolite, using an enzyme immunoassay (Amersham, Chicago Heights, IL).

Prior to performing the enzyme immunoassay, media aliquots were subjected to solid-phase extraction. This purification step was employed to isolate a fraction containing 6-prostaglandin $F_{1\alpha}$ free from nonesterified fatty acids which may interfere with the immunoassay determination of prostaglandins (40). Following acidification to pH 3 with 6 N hydrochloric acid, the samples were applied to solid-phase extraction columns containing C_2 packing. The prostaglandin fraction was eluted from the column with 5 mL of methyl formate after washing with 5 mL of methanol, 10% ethanol in water, then hexane, respectively.

Statistical analysis. The influence of CFAM treatment on the mean responses for the various biological parameters was tested for statistical significance using one-way analysis of variance. For each endpoint, the treatment means were compared in pairs using the Student's *t* test. Statistical probability of $P < 0.05$ was considered significant.

RESULTS

Cytotoxicity and membrane lipid composition. The cytotoxicity of CFAM was determined in cultured endothelial cells. As measured by the exclusion of trypan blue dye, viability of

TABLE 1
Composition of Lipid Materials Isolated from Cultured Porcine Aortic Endothelial Cells Incubated with or without CFAM–BSA (for 48 h)[a]

Parameter	Added CFAM–BSA (ppm)		
	0	31	62
Total lipid extract (mg/10^6 cells)	0.41 (0.02)	0.38 (0.04)	0.33 (0.04)
Polar lipid fraction (mg/10^6 cells)	0.35 (0.03)	0.33 (0.02)	0.31 (0.02)
Nonpolar lipid fraction (mg/10^6 cells)	0.06 (0.01)	0.05 (0.02)	0.02 (0.02)
Total cholesterol (μmol/10^6 cells)	0.10 (0.01)	0.10 (0.01)	0.10 (0.02)
Cholesterol-to-phospholipid (μmol/mg)	0.28 (0.04)	0.30 (0.03)	0.34 (0.04)
Phospholipid-to-protein (mg/mg)	0.98 (0.08)	0.98 (0.05)	0.80 (0.07)
Phospholipid class (wt)%			
Phosphatidylcholine	62.0 (2.1)	61.6 (1.7)	60.3 (1.6)
Phosphatidylethanolamine	31.5 (1.7)	32.2 (1.0)	34.5 (1.5)

[a]Values represent mean (± SD) for a single determination using two samples per treatment group; CFAM–BSA, cyclic fatty acid monomer–bovine serum albumin.

confluent endothelial cells was not significantly changed following treatment with BSA or CFAM–BSA media during the 48-h incubation interval.

The amount of material from the nonpolar and polar fractions of endothelial cell total lipid extracts was determined along with total cholesterol, enabling the ratios of cholesterol-to-phospholipid, cholesterol-to-protein, and phospholipid-to-protein to be calculated (Table 1). Additionally, the distribution of the major phospholipid classes isolated in the polar lipid fraction was determined (Table 1). The treatment of endothelial cells with CFAM–BSA did not cause significant change in the amount of total, nonpolar or polar lipid fractions, the distribution of phospholipid classes, or the cholesterol-to-phospholipid ratio. However, the ratio of phospholipid-to-protein decreased in cells treated with 62 ppm CFAM–BSA.

The incorporation of CFAM did not result in a substantial change of the relative amounts of unsaturated and saturated fatty acids. Using the ratio of total-unsaturated-to-total-saturated fatty acids, values of 1.6, 1.7, and 1.6 were determined for the control, 31 ppm CFAM–BSA, and 62 ppm CFAM–BSA groups, respectively. Upon CFAM–BSA supplementation, cultured endothelial cells incorporated CFAM into membrane phospholipids as well as neutral lipids. The fatty acid composition of the membrane and neutral lipids from endothelial cells revealed that the relative amount of CFAM within the membrane phospholipids from treatment with 31 and 62 ppm CFAM–BSA reached 3.2 and 4.3%, respectively (Table 2). CFAM containing cyclopentyl rings were preferentially incorporated into both membrane phospholipids and neutral lipids (Table 3). The polar and nonpolar lipid fractions exhibited identical CFAM GLC peak patterns to the pure fraction of hydrogenated CFAM (not shown).

Membrane lipid packing order. The steady-state anistropy (r) of DPH (Fig. 1) was found to decrease significantly in cul-

TABLE 2
Fatty Acid Composition (wt%) of M-199 Containing 5% FBS and the Nonpolar (NP) and Polar (P) Lipid Fractions Isolated from Cultured Porcine Aortic Endothelial Cells Incubated with or without CFAM–BSA (for 48 h)[a]

Fatty acid	M-199 + 5% FBS	Added CFAM–BSA (ppm)					
		0		31		62	
		NP	P	NP	P	NP	P
14:0	3.1	1.5 (0.2)	0.8 (0.1)	1.3 (0.2)	0.7 (0.1)	1.2 (0.1)	0.7 (0.1)
14:1	1.2	0.4 (0.1)	0.4 (0.1)	0.3 (0.1)	0.4 (0.1)	0.3 (0.1)	0.3 (0.1)
16:0	26.6	18.7 (0.5)	19.0 (0.7)	17.3 (0.5)	20.2 (0.8)	16.5 (0.6)	20.5 (0.4)
16:1	1.4	2.1 (0.1)	1.0 (0.1)	2.4 (0.3)	0.6 (0.1)	1.8 (0.1)	0.5 (0.1)
18:0	39.4	23.4 (0.6)	17.1 (0.1)	24.3 (0.4)	14.4 (0.7)	21.4 (0.7)	14.4 (0.5)
∑18:1	18.1	21.8 (0.5)	34.6 (0.1)	21.2 (0.2)	32.2 (0.6)	20.9 (0.1)	30.3 (0.5)
18:2	1.4	2.0 (0.2)	5.5 (0.3)	4.2 (0.2)	6.2 (0.1)	4.0 (0.2)	7.4 (1.0)
18:3	0.7	2.0 (0.3)	0.6 (0.1)	1.4 (0.1)	0.5 (0.1)	1.3 (0.1)	0.9 (0.1)
20:3	n.d.	2.3 (0.1)	1.1 (0.1)	1.9 (0.2)	1.0 (0.1)	1.4 (0.1)	1.2 (0.1)
20:4	8.7	5.4 (0.2)	7.7 (0.5)	3.2 (0.1)	5.6 (0.2)	2.6 (0.4)	4.8 (0.9)
22:4	n.d.	5.9 (0.5)	3.6 (0.4)	4.0 (0.5)	3.2 (0.2)	2.6 (0.3)	2.8 (0.5)
22:5	n.d.	6.6 (0.2)	3.9 (0.6)	4.2 (0.1)	3.3 (0.1)	2.7 (0.2)	2.9 (0.6)
22:6	n.d.	6.0 (0.4)	3.1 (0.7)	3.0 (0.4)	2.8 (0.1)	1.8 (0.1)	2.2 (0.5)
H_2 CFAM	n.d.	n.d.	n.d.	10.3 (0.6)	3.2 (0.3)	20.5 (0.8)	4.3 (0.4)

[a]Values represent mean (± SD) for a single determination using two samples per treatment group. H_2 represents hydrogenated. n.d. represents not detected. FBS, fetal bovine serum. See Table 1 for other abbreviations.

TABLE 3
CFAM Identified Following Hydrogenation of Lipid Fractions of Cultured Porcine Aortic Endothelial Cells

	Relative proportions of the major CFAM types[a] (% of total CFAM)					
	1	2	3	4	5	6
H_2 CFAM fraction	17.6	22.8	7.6	22.6	6.9	22.5
Polar lipid fraction	24.6	36.3	10.4	14.1	7.3	7.3
Nonpolar lipid fraction	35.4	38.0	—	16.1	6.3	4.1

[a]Numbers correspond to hydrogenated linseed oil CFAM structures. The structures are: 1 and 3, methyl 9-(2′-butylcyclopentyl)nonanoate; 2 and 5, methyl 10-(2′-propylcyclopentyl)decanoate; 4 and 6, methyl 9-(2′-propylcyclohexyl)nonanoate. Values represent single determinations. See Table 1 for abbreviations.

tured endothelial cells following the exposure to CFAM–BSA at both treatment levels compared to BSA controls ($P < 0.05$). The decrease was more significant by treatment with 62 ppm CFAM–BSA than 31 ppm ($P < 0.05$). Since lipid packing order is directly related to *r*, the lipid packing order of the hydrophobic core region decreased as a result of CFAM incorporation into endothelial cell membrane lipids as measured by DPH. This perturbation in order of the bilayer core was not reflected in lipid chain segments at or near the lipid–water interface of the membrane bilayer as shown by the lack of change in the *r* of DPH–PA (Fig. 2) and TMA–DPH (Fig. 3).

Presence of intracellular triglycerides. The presence of intracellular triglycerides using Nile red dye visualization was observed in cultured endothelial cells following incubation with 0, 31, and 62 ppm CFAM–BSA media for 48 h (Fig. 4). The location of intracellular lipid in the 0 ppm CFAM–BSA and BSA controls remained primarily around the nuclei of endothelial cells. Treatment with CFAM–BSA appeared to cause the formation of intercellular lipid droplets, but a loss of lipid around the nuclei of endothelial cells was observed (Fig. 4).

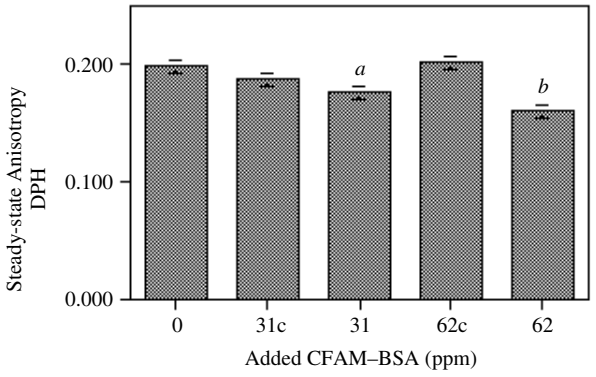

FIG. 1. Steady-state anisotropy (*r*) of 1,6-diphenyl-1,3,5-hexatriene (DPH) in confluent endothelial cells following exposure to media containing cyclic fatty acid monomer–bovine serum albumin (CFAM–BSA) for 48 h. Values represent mean ± SEM for triplicate determinations using three samples per treatment group; c indicates media containing an equivalent level of fatty acid-free BSA in the corresponding CFAM–BSA media; *a* indicates 31 ppm CFAM–BSA treatment group significantly different from 31c and 0 ppm CFAM–BSA treatment groups ($P < 0.05$); *b* indicates 62 ppm CFAM–BSA treatment group significantly different from 62c, 0 ppm, and 31 ppm CFAM–BSA treatment groups ($P < 0.05$).

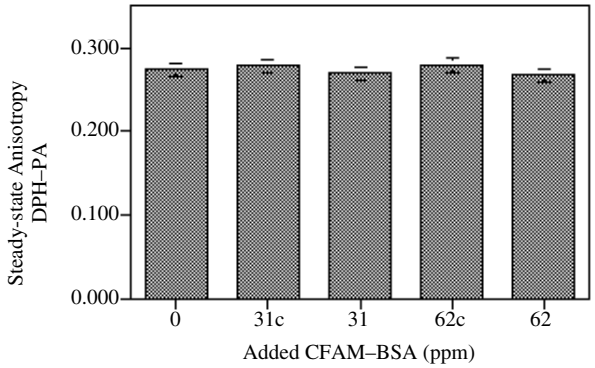

FIG. 2. Steady-state anisotropy (*r*) of DPH–propionic acid (PA) in confluent endothelial cells following exposure to media containing CFAM–BSA for 48 h. Values represent mean ± SEM using three samples per treatment group; c indicates media containing an equivalent level of fatty acid-free BSA as contained in the corresponding CFAM–BSA media. See Figure 1 for other abbreviations.

Monolayer integrity. Exposure to CFAM–BSA resulted in an increase of albumin movement across cultured endothelial cell monolayers (Fig. 5). Both levels of CFAM–BSA caused a significant increase in albumin movement vs. the 0 ppm CFAM–BSA and BSA control treatments ($P < 0.05$) with the influence of 62 ppm CFAM–BSA being more pronounced than 31 ppm ($P < 0.05$).

Calcium ATPase function. The activity of calcium ATPase decreased significantly ($P < 0.05$) in cultured endothelial cells treated with CFAM–BSA compared to the respective BSA controls (Fig. 6). The magnitude of the decreased ATPase activity corresponded to an increase in the level of CFAM–BSA in the media.

Prostacyclin synthesis. The production of 6-keto-prostaglandin $F_{1\alpha}$ was determined in cultured porcine aortic endothelial cells as a function of the incubation time and media concentration of CFAM–BSA (Table 4). The presence of CFAM–BSA in the media caused a significant increase in the production of prostacyclin compared to BSA alone. The production of prostacyclin was inhibited significantly owing to

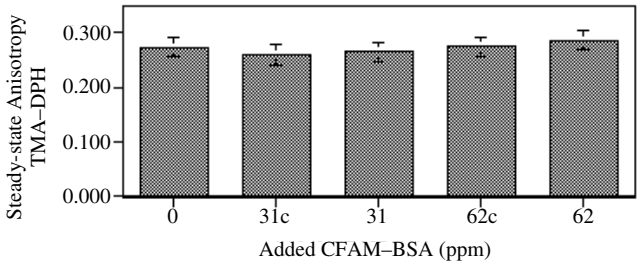

FIG. 3. Steady-state anisotropy (*r*) of 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA–DPH) in confluent endothelial cells following exposure to media containing CFAM–BSA for 48 h. Values represent mean ± SEM using three samples per treatment group. c indicates media containing an equivalent level of fatty acid-free BSA in the corresponding CFAM–BSA media. See Figure 1 for other abbreviations.

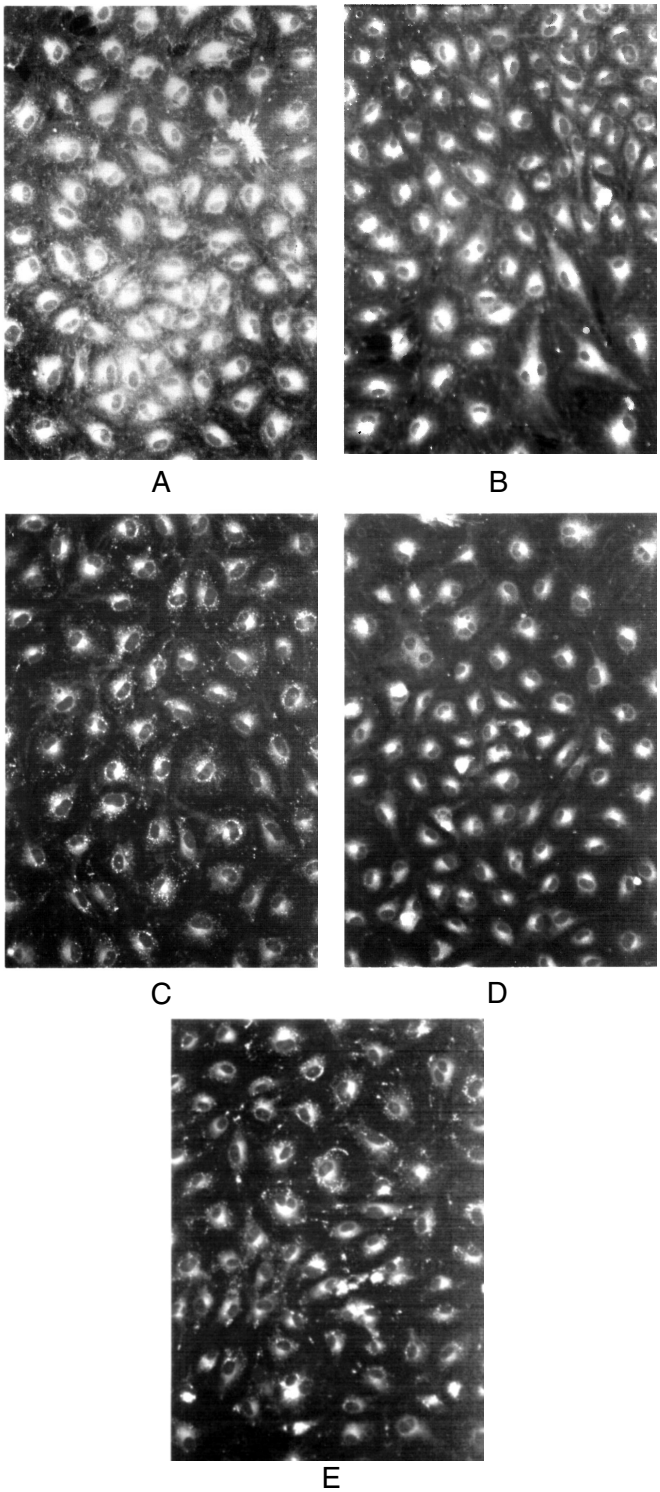

FIG. 4. Visualization by Nile red dye of intracellular lipid droplets in cultured aortic endothelial cells following incubation with 0 ppm CFAM–BSA (A), 31c (B), 31 ppm CFAM–BSA (C), 62c (D), and 62 ppm CFAM–BSA (E) media for 48 h. Magnification equals 200×; c indicates media containing an equivalent level of fatty acid-free BSA in the corresponding CFAM–BSA media. See Figure 1 for abbreviations.

the inclusion of indomethacin, a potent cyclooxygenase inhibitor, at a concentration of 5 μM in CFAM–BSA media (data not shown).

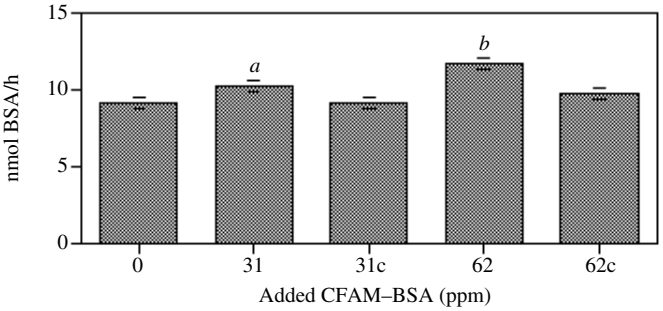

FIG. 5. Movement of BSA across confluent endothelial cell monolayers following exposure to media containing CFAM–BSA for 48 h. Values represent mean ± SEM using three samples per treatment group; c indicates media containing an equivalent level of fatty acid-free BSA as contained in the corresponding CFAM–BSA media; *a* indicates 31 ppm CFAM–BSA treatment group significantly different from 31c and 0 ppm CFAM–BSA treatment groups ($P < 0.05$); *b* indicates 62 ppm CFAM–BSA treatment group significantly different from 62c, 0 ppm, and 31 ppm CFAM–BSA treatment groups ($P < 0.05$). See Figure 1 for abbreviations.

DISCUSSION

The incorporation of CFAM into cellular membranes led to changes in several of the biochemical and physical parameters measured in confluent cultured endothelial cells. The uptake of cyclopentyl ring-containing CFAM was favored over cyclohexyl ring-containing CFAM in both the polar and nonpolar lipid fractions. This preferential uptake was observed in cells treated with both 32 and 61 ppm CFAM–BSA for 48 h. A similar trend has been described for the uptake of CFAM in cultured rat myocardiocytes (29).

Though not reported, we observed that CFAM–BSA and the BSA control media inhibited the growth and altered the morphology of cultured endothelial cells at both CFAM con-

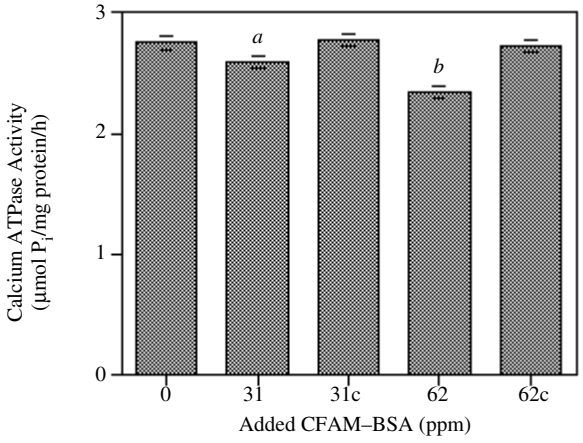

FIG. 6. Calcium ATPase activity of confluent endothelial cells following exposure to media containing CFAM–BSA for 48 h. Values represent mean ± SEM using three samples per treatment group; c indicates media containing an equivalent level of fatty acid-free BSA in the corresponding CFAM–BSA media; *a* indicates 31 ppm CFAM–BSA treatment group significantly different from 31c and 0 ppm CFAM–BSA treatment groups ($P < 0.05$); *b* indicates 62 ppm CFAM–BSA treatment group significantly different from 62c, 0 ppm, and 31 ppm CFAM–BSA treatment groups ($P < 0.05$). See Figure 1 for abbreviations.

TABLE 4
CFAM–BSA Time- and Concentration-Induced Release of 6-Keto Prostaglandin $F_{1\alpha}$ from Confluent Cultured Porcine Aortic Endothelial Cells[a]

CFAM–BSA (ppm)	6-Keto Prostaglandin $F_{1\alpha}$ (pg/mL)			
	4 H	8 H	12 H	24 H
0	402	461	531	855
31	308	447	481	796[b]
31c	—	213	417	331[a]
62	318	310[a,c,d]	332[a,d]	539[a,c,d]
62c	—	131	354	120[a]

[a]Values represent mean ± SD using two samples per treatment group. c indicates media containing an equivalent level of fatty acid-free BSA as contained in the corresponding CFAM–BSA media.[a]Treatment group significantly different from 0 ppm CFAM–BSA ($P < 0.05$). [b]31 ppm CFAM–BSA treatment group significantly different from 31c treatment group ($P < 0.05$). [c]62 ppm CFAM–BSA treatment group significantly different from 62c treatment group ($P < 0.05$). [d]62 ppm CFAM–BSA treatment group significantly different from 31 ppm CFAM–BSA treatment group ($P < 0.05$). See Table 1 for abbreviations.

centrations. Inhibition of growth and morphological changes in cultured cells by supplementation with the nonurea-adducting fraction isolated from thermally abused oils have been reported (41). These authors combined the fatty acids from fresh and heated oils with fatty acid-free BSA (1:60 w/w or 4:1 molar ratio) then added the complexed fatty acids–BSA to the media at concentrations of 20, 60, and 100 mg/L (ppm). The fatty acids from fresh oils served as controls for the measurement of cell growth and determination of changes in cell morphology. The use of BSA controls was not reported. It is our contention that BSA should not be used to incorporate fatty acids into media preparations when examining the influence of fatty acids on cell growth and morphology.

The present experimental evidence suggests that CFAM can alter specific physical characteristics of cellular membranes as well as biological processes involved in the maintenance of homeostatic functions in cultured endothelial cells. In examining factors which influence membrane lipid packing order, the lipid parameters which have undergone measurable change are the phospholipid-to-protein ratio and the presence of CFAM in the membrane lipids. The phospholipid-to-protein ratio and membrane lipid packing order have been observed to be directly proportional (42,43). In addition to further increase in membrane CFAM content compared to the 31 ppm CFAM–BSA treatment, the 62 ppm CFAM–BSA treatment caused a decreased cellular phospholipid-to-protein ratio which may contribute to the observed decrease in membrane lipid packing order.

Another factor to be considered in determining lipid packing order is accumulation of intracellular triglycerides. The use of DPH polarization studies in culture systems often has been precluded owing to the formation of excessive amounts of intracellular triglycerides caused by supplementation of exogenous fatty acids. The uptake of DPH by intracellular lipid droplets in intact cells contributes to a marked decrease in the steady-state anistropy of DPH (44) while the *r* of TMA–DPH and DPH–PA is not influenced by accumulation of intracellular lipid droplets (45). Membrane lipid packing order may not be markedly affected by exposure of cultured endothelial cells to CFAM–BSA as suggested by increased intracellular triglycerides caused by CFAM–BSA exposure as visualized with the use of Nile Red dye. However, the amount of lipid isolated in the nonpolar fraction, which is predominantly triglycerides, was not significantly different between cells treated with and without CFAM–BSA.

The observation that calcium ATPase activity was decreased by exposure of cells to CFAM stands in contrast to the influence of linolenic acid which causes an increase in the activity of total ATPase in lymphocytes (46) and calcium ATPase in endothelial cells (8). This may suggest that the ring structure present in CFAM elicits the same effect on ATPase activity observed for cholesterol incorporation into kidney fibroblast membranes (47). Preliminary results suggest that CFAM treatment also causes a decrease in the total ATPase activity.

The effect of CFAM on monolayer integrity of cultured endothelial cells was identical to the influence of linoleic acid in various mixtures of fatty acids including those prepared from selected edible oils (8). Our result suggests that CFAM disrupt the integrity of the monolayer through changes in cell–cell interactions or transcytosis. Incubation with CFAM–BSA may cause morphological changes in cultured endothelial cells leading to disruption of the monolayer. However, phase-contrast microscopy was the only indicator of endothelial cell morphology used for the observation of the endothelial cell monolayer in culture-well inserts.

CFAM caused greater production of prostaglandin I_2 (PGI_2) (prostacyclin) with respect to the BSA controls, suggesting either that the presence of CFAM was counteracting the inhibitory influence of BSA on PGI_2 production or that CFAM were directly stimulating PGI_2 secretion by cultured endothelial cells. In light of the fact that both BSA and CFAM–BSA caused a decreased production of PGI_2 with respect to the 0 ppm CFAM–BSA media, the presence of CFAM may be compensating for the action of BSA.

Despite the fact that CFAM represented only 8% of the total lipids in the culture medium, the incorporation of CFAM from heated linseed oil into the membrane of cultured endothelial cells caused specific changes in several of the parameters examined in this study. This experimental data indicate that CFAM are by no means inert constituents of biological membranes but can exhibit potent physiological effects *in vitro*. In fact, it is possible that the long-term presence of CFAM in diets may influence the susceptibility of the vascular endothelium toward injury and degeneration. However, having so speculated, the presence of CFAM in membrane phospholipids of vascular endothelia has yet to be confirmed *in vivo*. The present results indicate the need for further consideration regarding the potential impact associated with the presence of CFAM in the diet.

ACKNOWLEDGMENTS

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The Effect of Highly Purified Eicosapentaenoic and Docosahexaenoic Acids on Monocyte Phagocytosis in Man

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ABSTRACT: The n-3 fatty acids (FA) from marine sources are known to exert antiinflammatory effects on monocyte function. There is still controversy whether n-3 FA may increase the susceptibility to infections. The present study was designed to assess the effect of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on monocyte phagocytosis and respiratory burst activity. Fifty-eight healthy men were randomized to take a daily supplement of 3.8 g highly purified EPA (n = 20), 3.6 g DHA (n = 19), or corn oil (n = 19) for 7 wk. Mononuclear leukocytes were collected, isolated, and cryopreserved prior to and after dietary supplementation. Paired samples were analyzed in the presence of autologous serum in a crossover design. Monocyte phagocytosis and respiratory burst activity were measured by flow cytometry after ingestion of *Escherichia coli*. Monocytes retained their phagocytic ability and respiratory burst activity after supplementation. No reduction in internalization of bacteria was registered. Dietary n-3 FA and particularly EPA improved bacterial adherence to the monocyte surface. In the crossover experiments, there was an adverse effect of serum enriched with n-3 FA on bacterial adherence. We conclude that monocytes retain their phagocytic potential after supplementation with purified EPA and DHA.
Lipids 32, 935–942 (1997).

Monocytes play an important part in the defense against infections. They act as antigen-presenting cells, as producers of cytokines, and as phagocytes (1). In addition, they have protective functions by ingesting and killing invading microorganisms. Monocytes are particularly involved in chronic infections, such as tuberculosis and malaria, and in viral infections (2–5).

The n-3 fatty acids (FA) have been reported to decrease monocyte and neutrophil chemotaxis (6), decrease leukocyte superoxide generation (7,8), modify eicosanoid production, and decrease cytokine synthesis (9,10). Thus, n-3 FA may influence inflammatory and immune reactions which are involved in the development of disease. Populations who consume diets rich in n-3 FA have low incidence of inflammatory diseases (11), and fish oil enriched with eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) seems beneficial to patients suffering from such disorders (12,13). On the other hand, it has been suggested that polyunsaturated FA may encourage infection *via* attenuated inflammatory response and immunosuppression (14). Fish oil has been reported both to decrease and to improve survival rate of animals after experimentally induced infections (15–17).

Leukocyte phagocytosis is an important part of the host defense against infections, and few studies have examined the effect of n-3 FA on phagocytosis. In human neutrophils, the phagocytic process diminished (18) or remained unchanged (19) following n-3 FA supplementation. Both improved and decreased phagocytic ability have been reported in murine macrophages after dietary supplementation with n-3 FA (20,21).

Experimental studies indicate that EPA and DHA may have specific and partly different actions on cellular functions (22,23). Previous dietary studies have not investigated the effect of n-3 FA on monocyte phagocytosis, but have restricted their focus to neutrophils. We therefore examined the effect of dietary supplementation with highly purified EPA and DHA on monocyte phagocytosis and respiratory burst activity in humans *ex vivo*. Cryopreserved human monocytes isolated before and at the end of the supplementation period were used to avoid methodological variability (24), and the phagocytic properties were assessed with flow cytometry (FCM), allowing accurate measurements of single-cell functions on a large scale.

MATERIALS AND METHODS

Subjects and study design. Two hundred thirty-four healthy, nonsmoking men entered a double-blind parallel group intervention study lasting for 7 wk. Their mean age was 44 ± 5 yr

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Abbreviations: AT, acid-treated; CA, cryopreserved cells collected after intervention period; CB, cryopreserved cells collected before intervention period; CO, corn oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; FCM, flow cytometry; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; SA, serum prepared after supplementation period; SB, serum prepared before supplementation period.

(range 36–56). They did not take any drugs regularly, and consumed a traditional Western diet with less than four dishes of fish per week and did not expect to change their diet or lifestyle during the study period. Their total serum cholesterol was <8 mmol/L, diastolic blood pressure was <95 mm Hg, and systolic blood pressure was <160 mm Hg. The participants were randomly assigned to ingest either 4 g/d of 95% ethyl ester concentrate of EPA (20:5n-3), 4 g/d of 90% ethyl ester concentrate of DHA (22:6n-3), or 4 g/d of corn oil. The oils were given in indistinguishable soft gelatin capsules each containing 1 g oil and 3.0 IU vitamin E as antioxidant. Each person was asked to ingest four capsules a day. Drugs were manufactured by Pronova Biocare A/S (Oslo, Norway). Twenty subjects were randomly selected from each group prior to the intervention period and asked to give additional blood for isolation of mononuclear leukocytes before and after 7 wk of supplementation. Compliance was calculated as the percentage of the prescribed capsules taken. The study was approved by the regional board of research ethics and was performed according to Good Clinical Practice requirements (25). Written informed consent was obtained from all participants.

Serum. Autologous serum was obtained before and after 7 wk of supplementation with n-3 FA and prepared by clotting whole blood in sterile glass tubes for 1 h at room temperature and then centrifuged at 2000 × *g* for 10 min. Serum was frozen in aliquots of 0.5 mL and stored at −70°C.

Preparation of peripheral blood mononuclear cells. The isolation, freezing, and thawing procedures have been described previously (24). After overnight fasting, 120 mL blood was drawn from an antecubital vein into four 50-mL Falcon tubes (Becton Dickinson, Rutherford, NJ) containing EDTA as anticoagulant. Fifteen mL of the sample was mixed with 15 mL glucose-phosphate buffer and layered on top of 15 mL Lymphoprep (Nycomed Pharma, Oslo, Norway) and centrifuged at 301 × *g* for 30 min. The mononuclear cells at the interface were carefully pipetted off, transferred into another Falcon tube, and diluted before a second centrifugation step. The cell pellet was resuspended in 10 mL glucose-phosphate buffer and centrifuged slowly (20 × *g* for 15 min at 4°C) to remove the platelets. Thereafter the mononuclear leukocytes were resuspended and washed twice in RPMI 1640 (Biowhittaker, Walkersville, MD) containing 5% acid-treated (AT) fetal calf serum (FCS).

Freezing procedure. Mononuclear leukocytes suspended in 5% AT-FCS-RPMI were centrifuged, and the pellet was resuspended in precooled RPMI 1640 with 50% AT-FCS. An equal volume of ice-cold RPMI containing 20% dimethyl sulfoxide dried (Merck, Darmstadt, Germany) was added dropwise over 3 min. Aliquots of 1 mL (4×10^6 mononuclear leukocytes) were dispersed into sterile cryo vials (Greiner Labortechnik, Frickenhausen, Germany). The tubes were placed in an isopropylene box at room temperature, then frozen at −70°C for at least 2 h, and stored until further use at −135°C in a Bio-freezer (Queue Cryostar, Queque Systems, Parkenberg, WV).

Thawing procedure. The frozen cells were rapidly thawed with gentle and continuous agitation at 37°C in a water bath. The suspensions were transferred into 50-mL Falcon tubes in droplet form with 9 mL 20% FCS in RPMI 1640. The cells were centrifuged and finally resuspended in 5% heat-inactivated FCS in RPMI 1640. All experiments were performed 2 h after thawing. Mononuclear cell- and differential counts were made prior to and after thawing of the mononuclear leukocytes in a Coulter Counter S-plus STKR (Coulter Electronics, Harpenden, United Kingdom).

Flow cytometry. A Facscan flow cytometer (Becton Dickinson, San Jose, CA) with an excitation wavelength of 488 nm interfaced to a Hewlett-Packard 340 computer (Hewlett-Packard, Tokyo, Japan) was used for cell identification and measurements of monocyte phagocytosis and respiratory burst activity (24). By recording the fluorescence and light scattering of each cell, information relating to cell size and structure was determined. The resulting scattered light signals were given in a numerical scale and fluorescence signals in a logarithmic scale. Fluorescein isothiocyanate (FITC) fluorescence was measured at 530 nm, rhodamine at 500–530 nm, and right-angle light scatter at 488 nm. Calibration of light scattering and fluorescence was conducted daily with Calibrite beads (Becton Dickinson).

Bacteria. Heat-inactivated *Escherichia coli* (serotype O102:K52:H6, hemolysine negative) isolated from blood cultures were labeled with FITC (Sigma Chemical Company, St. Louis, MO). The number of bacteria were adjusted to 1.5×10^5 per sample. Unlabeled bacteria from the same strain were used in the respiratory burst experiments.

Phagocytosis. Opsonization was performed by incubating the bacterial suspension (1.5×10^6) with 0.5% autologous serum for 7.5 min on a rock'n roller at 37°C. Monocytes (5×10^4) were added, giving a bacteria–cell mixture of 250 µL, and after 5 min the reaction was stopped by adding 250 µL ice-cold phosphate buffered saline with 0.05% EDTA. Mononuclear leukocytes were differentiated by FCM along the right angle- and forward light scatter, and gated separately. Anti-CD14 mAb (Immunotech, Marseille, France) was used to identify the monocyte population and to verify correct monocyte gating. The gate was set manually. Nonphagocytes and phagocytes were differentiated by simultaneous measurements of right-angle light scatter and FITC fluorescence by FCM. Phagocytes expressing fluorescence due to attached and/or internalized FITC-labeled bacteria were quantified as the percentage of the monocyte population (10,000 monocytes). The amount of attached and/or internalized bacteria was quantified as mean fluorescence intensity (MFI) (24,26). An FCM fluorescence quenching technique was used to distinquish between attachment and internalization of bacteria by addition of trypan blue to the cell suspensions (27). Extracellular FITC fluorescence representing attached bacteria was quenched, whereas the fluorescence of internalized bacteria remained unchanged.

Respiratory burst activity. Monocytes (5×10^4) in 250 µL RPMI 1640 with 5% FCS were mixed with 100 µL (10

μg/mL) dihydrorhodamine (Molecular Probes, Eugene, OR) and incubated for 15 min at 37°C. Unlabeled bacteria (1.5×10^6) were opsonized with 0.5% autologous serum and internalized as described above. The fluorochrome accumulated in the cells and was oxidized intracellularly to fluorescent rhodamine after stimulation of the respiratory burst by exposure to bacteria. The fluorescence produced was proportional to the amount of generated hydrogen peroxide. Ten thousand monocytes were counted, and MFI of the whole monocyte population was measured by FCM.

Plasma phospholipid FA. FA were measured by extracting total lipids from 500 μL serum according to Folch *et al.* (28) with phosphatidylcholine diheptadecanoyl added as internal standard (P-5014; Sigma Chemical Co.), chloroform/methanol (2:1, vol/vol) as solvent and butylated hydroxytoluene (75 mg/L) as antioxidant. Total phospholipids were separated by solid-phase extraction using NH_2 columns (size 3 cc Analytic Bond Elut LRC; Varian, Harbor City, CA)(29), followed by transmethylation using boron trifluoride, extraction into hexane and evaporation to dryness. The FA methyl esters were dissolved in hexane and analyzed by gas–liquid chromatography (Shimadzu GC-14A; Shimadzu Co., Kyoto, Japan) using a capillary column (CP-Sil 88, length 50 m i.d. 0.25 mm) obtained from Chrompack Inc. (Raritan, NJ). Retention times and response factors for each FA were determined using standards obtained from Nu-Chek- Prep (Elysian, MN). The results were integrated on a Shimadzu C-R4A integrator. The FA concentrations are reported as μmol/L plasma.

Experimental design and statistical analysis. Mononuclear leukocytes were isolated and cryopreserved prior to and at the end of the supplementation period. Cryopreserved cells collected before (CB) and after (CA) the intervention period from the same participant were thawed and examined on the same day. Serum was also prepared before (SB) and after (SA) the supplementation period, allowing cells to be examined in the presence of autologous serum. Samples were analyzed blindly, and cryopreservation of cells allowed all assays for each subject to be run at the same time. The following experimental design was set up: (i) CB + SB, (ii) CA + SA, (iii) CB + SA, and (iv) CA + SB. The effect of supplementation on phagocytic functions in monocytes was assessed by comparing results obtained in these various conditions: (I) (CA + SB) minus (CB + SB); (II) (CA + SA) minus (CB + SA); (III); (CA + SA); minus (CB + SB); (IV) (CA + SB) minus (CB + SA). The experimental conditions were designed to differentiate between effects of intervention on cellular functions and on serum components.

Examination of the frequency distributions revealed normally distributed variables. Change was calculated as the value obtained after intervention minus the value obtained at baseline according to the above scheme for phagocytic variables. One sample *t*-tests were used to evaluate within-group change (condition III). Analysis of variance was used to evaluate overall difference between-groups (F-test). If the F-test was significant, between group comparisons of change were done by contrasting groups in the SAS general linear models procedure (30). Results were considered significant when the two-sided *P*-value was <0.05. Results are expressed as means ± standard deviation.

RESULTS

Table 1 shows that the groups randomized to receive EPA, DHA, or corn oil (CO) were well balanced at baseline. One subject in the DHA group withdrew after 4 d of supplementation due to gastrointestinal discomfort. One person in the CO group withdrew after 4 wk of supplementation due to acute vertigo and vomiting probably not related to the supplementation. Side effects (belching) were mild and transient, fading 1–2 wk after initiation of trial medication. Compliance was similar in all groups (Table 1). Body mass index (kg/m^2) increased in the EPA (0.3 ± 0.4, $P = 0.0027$) and the CO (0.2 ± 0.3, $P = 0.027$) group during supplementation, but the difference between groups was not significant.

Effects on phagocytosis. When all 58 participants were included in the analysis prior to dietary intervention, 15.5 ± 3.6% of the monocytes were associated with bacteria without opsonizing autologous serum. Introduction of serum increased the percentage of monocytes associated with bacteria to 43.6 ± 7.6%, which corresponded to an MFI of 817.7 ± 72.7. Addition of trypan blue, to extinguish the fluorescence

TABLE 1
Characteristics of Participants Receiving Docosahexaenoic Acid (DHA), Eicosapentaenoic Acid (EPA), or Corn Oil (CO)

	DHA	EPA	CO
Number	19	20	19
Age (yr)	42.9 (4.8)	43.9 (4.9)	43.9 (4.8)
BMI[a] (kg/m2)	25.1 (2.2)	24.7 (2.0)	24.6 (2.5)
SBP[b] (mm Hg)	122.3 (8.0)	123.4 (7.9)	122.8 (10.7)
DBP[c] (mm Hg)	76.6 (6.2)	77.5 (6.5)	78.3 (7.7)
Cholesterol (mmol/L)	5.98 (1.08)	5.78 (0.82)	6.08 (1.09)
Triglycerides (mmol/L)	1.38 (0.79)	1.20 (0.55)	1.17 (0.48)
Compliance[d] (%)	91.3 (7.9)	92.2 (5.7)	91.9 (11.0)

[a]BMI = body mass index. [b]SBP = systolic blood pressure. [c]DBP = diastolic blood pressure. [d]Percentage compliance was calculated as the percentage of the prescribed drugs taken. Values are mean (SD).

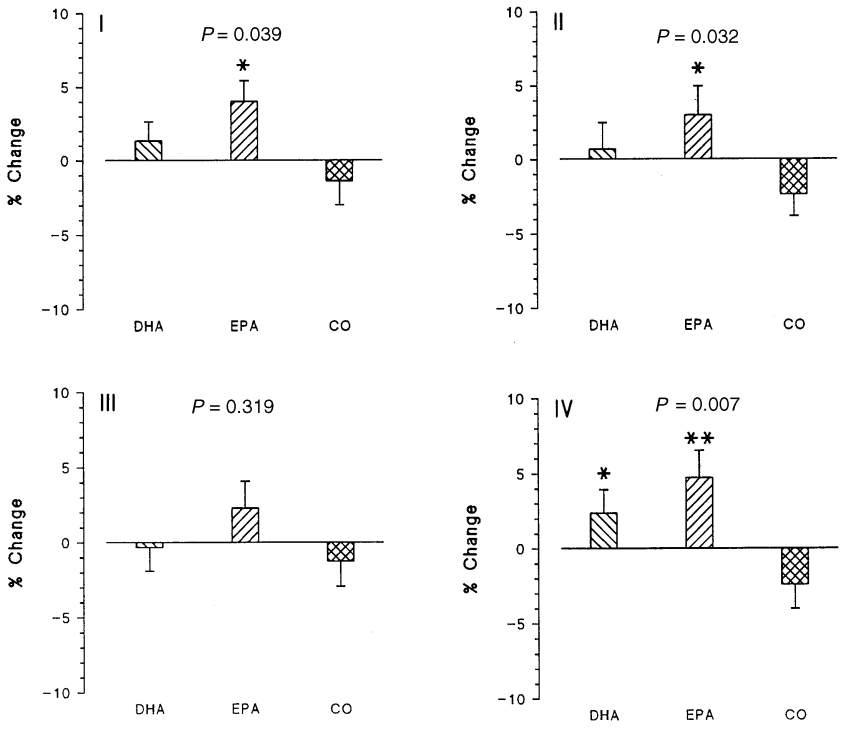

FIG. 1. Percentage change of attached and internalized *Escherichia coli* in monocytes from participants receiving dietary supplementation with purified docosahexaenoic (DHA) ($n = 19$) or eicosapentaenoic (EPA) ($n = 20$) acids, or corn oil (CO) ($n = 19$) for 7 wk. Cryopreserved cells collected before (CB) and after (CA) intervention were examined in the presence of autologous serum collected before (SB) or after (SA) intervention. The effect of supplementation was assessed according to the following model; (I) (CA + SB) minus (CB + SB), (II) (CA + SA) minus (CB + SA), (III) (CA + SA) minus (CB + SB), and (IV) (CA + SB) minus (CB + SA). The *P*-values for the overall F-test are given for each experimental condition. Stars indicate significant difference from CO. * $P < 0.05$ and ** $P < 0.01$. Bars are mean ± SD.

signal from attached and not internalized bacteria, decreased MFI by 50.7 ± 7.2%. Thus, under these experimental conditions, approximately 50% of the associated bacteria were actually internalized.

Phagocytosis of unopsonized bacteria revealed no differences between groups regarding percentage of phagocytosing monocytes nor the amount of associated bacteria (reflected by MFI)(data not shown).

In the presence of autologous serum (Fig. 1 and Table 2), EPA increased the cellular attachment of bacteria in the presence of a constant serum component, which differed significantly from CO (I, $P = 0.01$ and II, $P = 0.03$, respectively), but not from DHA ($P = 0.24$ and $P = 0.36$, respectively). In condition III, which reflects the physiological milieu with cells and serum collected simultaneously, the change in bacterial attachment within the EPA group was diminished, but remained significant ($P = 0.015$). The change did not differ from the other groups. However, when cells collected after intervention were incubated with serum collected before, the enhancement in bacterial attachment was reinforced, and both

TABLE 2
Human Monocyte Phagocytosis of *Escherichia coli* from Participants Receiving Dietary Supplementation with DHA ($n = 19$), EPA ($n = 20$), or CO ($n = 19$) for 7 wk[a]

	DHA		EPA		CO	
	MFI	Attachment (%)	MFI	Attachment (%)	MFI	Attachment (%)
CB + SB	824.3 ± 58.3	49.5 ± 7.4	795.3 ± 80.7	51.3 ± 6.8	834.9 ± 74.4	51.1 ± 7.6
CB + SA	817.6 ± 71.9	49.7 ± 7.4	789.6 ± 72.9	52.1 ± 6.8	845.3 ± 93.7	51.9 ± 8.3
CA + SB	834.4 ± 63.4	53.9 ± 7.5	825.5 ± 77.6	55.2 ± 7.5	820.8 ± 62.8	54.4 ± 7.4
CA + SA	821.1 ± 72.4	55.1 ± 7.8	811.8 ± 86.9	56.2 ± 7.3	822.3 ± 72.2	54.9 ± 6.9

[a]Phagocytosis is expressed as mean fluorescence intensity (MFI) of attached and internalized bacteria and percentage of attached bacteria. Cryopreserved cells collected before (CB) and after (CA) intervention were examined in the presence of autologous serum collected before (SB) or after (SA) intervention. See Table 1 for other abbreviations.

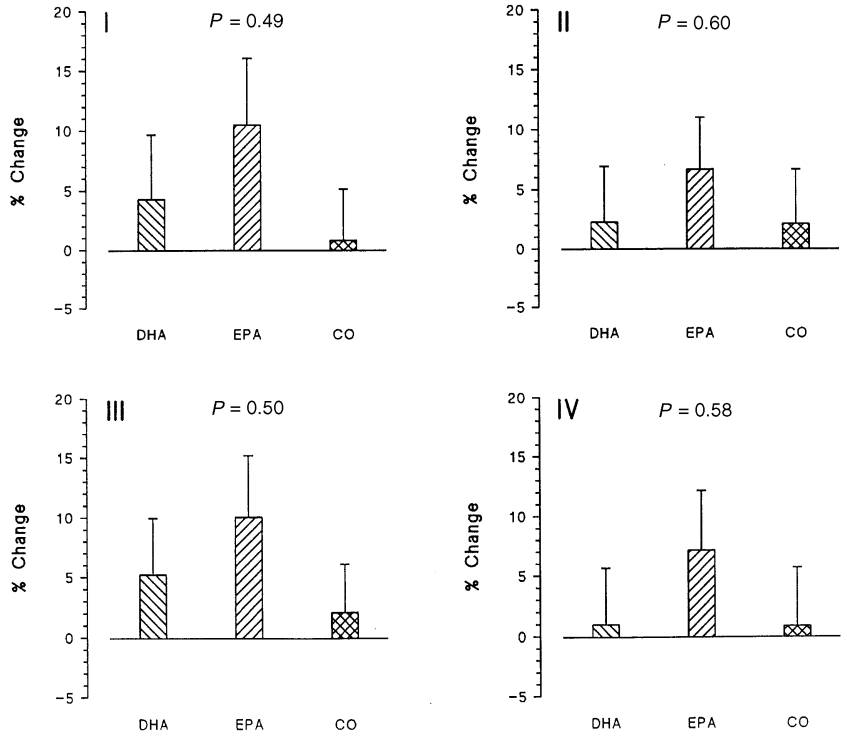

FIG. 2. Percentage change of respiratory burst activity in monocytes exposed to opsonized *E. coli*, from participants receiving dietary supplementation with purified DHA (n = 19) or EPA (n = 20) acids, or CO (n = 19) for 7 wk. Cryopreserved cells collected before (CB) and after (CA) intervention were examined in presence of autologous serum collected before (SB) or after (SA) intervention. The effect of supplementation was assessed according to the following model: (I) (CA + SB) minus (CB + SB), (II) (CA + SA) minus (CB + SA), (III) (CA + SA) minus (CB + SB), and (IV) (CA + SB) minus (CB + SA). The *P*-values for the overall F-test are given for each experimental condition. Bars are mean ± SD. See Figure 1 for other abbreviations.

DHA (P = 0.03) and EPA (P = 0.002) differed significantly from CO. Addition of trypan blue to extinguish the fluorescence signal from attached bacteria abolished all significant changes between the groups described above (data not shown).

Effects on respiratory burst activity. Percentage change of intracellularly generated hydrogen peroxide after exposure to *E. coli* is visualized in the three intervention groups under four experimental conditions (Fig. 2 and Table 3). The generation of hydrogen peroxide tended to be enhanced within the EPA group under all experimental conditions, but the P values for the overall F-tests were not significant under any experimental condition. However, the pattern was similar to what was observed in the phagocytosis experiments with a tendency toward an increase in the EPA and DHA groups compared to CO.

Plasma phospholipid FA concentrations. During intervention there was a small increase in plasma phospholipid saturated FA in the CO group (4%, $P < 0.05$), as compared to no change in the EPA and DHA groups (Table 4). The monounsaturated FA decreased slightly in both the DHA (–15%, $P < 0.01$) and EPA (–9%, not significant) groups. The total amount of plasma phospholipid polyunsaturated FA did not change in the DHA and CO groups, whereas there was a 9% decrease ($P < 0.001$) in the EPA group. This was due to a relatively larger decrease in linoleic (–26%, $P < 0.001$) and

TABLE 3
Respiratory Burst Activity in Human Monocytes Exposed to Opsonized Bacteria[a]

	DHA	EPA	CO
CB + SB	35.7 ± 8.8	32.2 ± 9.1	35.2 ± 10.4
CB + SA	35.9 ± 6.7	34.6 ± 9.4	34.8 ± 9.2
CA + SB	37.1 ± 11.1	33.2 ± 9.4	35.6 ± 11.1
CA + SA	36.3 ± 6.2	34.8 ± 10.5	35.3 ± 8.8

[a]Expressed as mean fluorescence intensity from participants receiving dietary supplementation with purified DHA (n = 19), EPA (n = 20), or CO (n = 19) for 7 wk. Cryopreserved cells collected before (CB) and after (CA) intervention were examined in the presence of autologous serum collected before (SB) or after (SA) intervention. See Table 1 for other abbreviations.

TABLE 4
Plasma Phospholipid Fatty Acid Concentration (μmol/L) Before and After 7 wk of Supplementation with DHA, EPA, or CO[a]

Fatty acid	DHA (n = 19)		EPA (n = 20)		CO (n = 19)		F-test[b]	Contrasts between groups P		
	Baseline	Week 7	Baseline	Week 7	Baseline	Week 7	P	DHA vs. EPA	DHA vs. CO	EPA vs. CO
Saturated	2127 ± 317	2068 ± 256	2037 ± 245	1975 ± 256	2100 ± 387	2193 ± 493[c]	0.03	0.9	0.02	0.02
Monounsaturated	441 ± 97	377 ± 83[d]	435 ± 109	398 ± 108	429 ± 94	443 ± 119	0.02	0.3	0.006	0.06
Polyunsaturated	1454 ± 334	1474 ± 262	1539 ± 195	1401 ± 199[e]	1609 ± 235	1667 ± 349	0.0008	0.003	0.5	0.0004
n-3	237 ± 87	379 ± 135[e]	268 ± 78	463 ± 108[e]	326 ± 107	315 ± 134	0.0001	0.08	0.0001	0.0001
Linoleic 18:2n-6	843 ± 196	795 ± 109	896 ± 151	660 ± 113[e]	882 ± 160	937 ± 186[d]	0.0001	0.0001	0.007	0.0001
Arachidonic 20:4n-6	252 ± 78	209 ± 50[d]	259 ± 68	198 ± 48[e]	276 ± 64	279 ± 71	0.0001	0.2	0.0008	0.0001
Eicosapentaenoic 20:5n-3	40.0 ± 19.2	57.1 ± 21.9[d]	48.4 ± 23.4	231.6 ± 66.7[e]	68.9 ± 44.0	53.6 ± 32.7	0.0001	0.0001	0.04	0.0001
Docosapentaenoic 22:5n-3	29.4 ± 12.0	17.3 ± 5.7[e]	34.1 ± 10.1	80.4 ± 28.0[e]	37.0 ± 10.5	38.8 ± 14.2	0.0001	0.0001	0.01	0.0001
Docosahexaenoic 22:6n-3	159 ± 60	298 ± 110[e]	176 ± 53	144 ± 40[d]	214 ± 67	215 ± 91	0.0001	0.0001	0.0001	0.06

[a]Values are means ± SD. See Table 1 for abbreviations.
[b]Analysis of variance for between-group comparisons of change.
[c]$P < 0.05$; [d]$P < 0.01$; [e]$P < 0.001$; one sample t-test of difference between baseline and 7 wk.

arachidonic acid (−24%, $P < 0.001$) in the EPA group as compared to the DHA group (linoleic acid −6%, not significant; arachidonic acid −17%, $P < 0.01$). In the CO group, plasma phospholipid linoleic acid concentration increased by 6% ($P < 0.01$). During intervention, plasma phospholipid concentration of n-3 FA increased by 60% in the DHA group and by 73% in the EPA group (both $P < 0.001$). In the DHA group, plasma phospholipid DHA and EPA concentrations increased by 87 and 43%, respectively, indicating retroconversion of DHA to EPA, whereas docosapentaenoic acid decreased by 41% ($P < 0.001$). In the EPA group, plasma phospholipid EPA increased by 379% and docosapentaenoic acid by 136% (both $P < 0.001$), whereas DHA decreased by 18% ($P < 0.01$).

DISCUSSION

The n-3 FA from marine sources are reported to modify eicosanoid production and to decrease cytokines and superoxide generation in human studies (7–10). Despite such apparently immunosuppressive actions of n-3 FA, the present study shows that monocytes retain the phagocytic potential and improve their ability to attach bacteria after supplementation with EPA. No reduction in internalization of bacteria was registered. Similar but less prominent changes were observed after supplementation with DHA. The observed changes are caused by altered monocyte function, because bacterial attachment increased in experiments with a constant serum component.

The n-3 FA exert their immunoregulatory effects through modification of the FA composition in membranes of immunocompetent cells. EPA is more effectively incorporated into monocyte membranes than DHA (9,10), and such incorporation occurs within a short period of time, in which there is a direct exchange of phospholipid FA between plasma and cells (31,32). Our data show that EPA is more readily incorporated into plasma phospholipid FA than DHA, which may reflect similar changes in monocyte membrane FA.

Phagocytosis is a rapid, energy-demanding process involving attachment and internalization of the phagocytic particle. Effective engulfment is promoted through complement and immunoglobulin receptors. Whether such opsonin receptor expression is modulated during n-3 FA supplementation is unclear, but decreased expression of adhesion molecules has been reported (21,33). These adhesion molecules are particularly involved in monocyte–endothelium interactions, but are also associated with bacterial adherence to phagocytes. Furthermore, monocytes possess an nonspecific, opsonin-independent pathway for attachment and internalization of bacteria, in which membrane fluidity seems important for the presentation of the synthesized ligand receptors (34). The n-3 FA, and particularly EPA may increase the monocyte membrane fluidity (20,33) and thereby influence the expression of such membrane proteins.

The enhanced bacterial attachment was attenuated in experiments with cells and serum collected simultaneously, but reinforced in the crossover experiments for both EPA and

DHA. Our data therefore indicate that serum enriched with n-3 FA may have an adverse effect on bacterial attachment. Owing to a diluted serum component (10%) and short incubation time of serum with monocytes, it is unlikely that n-3 enriched serum could affect membrane fluidity *in vitro*. However, reactive lipid peroxides generated from blood cells during serum preparation are possibly modified by the n-3 FA supplementation and may thereby affect monocyte behavior. Another possibility is that n-3 FA interfere with serum opsonins such as complement factors and immunoglobulins.

Conflicting data have been reported regarding the influence of unsaturated FA on leukocyte phagocytosis. Few studies are comparable, as both marine and vegetable FA with different degrees of unsaturation and purity have been studied in the presence of various artificial and natural agents. Our data indicate that neither highly purified n-3 FA nor n-6 FA seemed to affect internalization of bacteria. This is consistent with data reported by Knapp and Fitz-Gerald (19), showing that neutrophil phagocytosis was not altered after a daily intake of 10 g/day of EPA for 4 wk. In contrast, Virella *et al.* (18) observed that neutrophil phagocytosis of latex beads was depressed during supplementation with 2.1 g/d of EPA for 6 wk in a single person. In animal studies, both increased and depressed macrophage phagocytosis have been reported after n-3 FA supplementation (20,21). Calder *et al.* (20) concluded that the *in vitro* phagocytic activity increased with increasing degree of unsaturation, except for DHA and EPA which showed lower activity than linoleic (18:2n-6), linolenic (18:3n-3), and arachidonic (20:4n-6) acids. In contrast, Eicher and McVey (21) described depressed *ex vivo* phagocytic activity in fish oil-fed mice during severe salmonella infection. It is difficult to interpret these results, as macrophages are located in tissues for several weeks compared to the short circulation time of monocytes and neutrophils.

Phagocytosis has been reported to be impaired by medium-chain triglycerides, but not in presence of long-chain triglycerides (35). Thus, one might speculate whether the observed changes in bacterial attachment are secondary to reduced serum triglyceride levels. However, *in vivo* studies have not confirmed an inverse association between triglycerides and neutrophil phagocytosis (36,37). In the present study there was no association between changes in serum triglycerides and alterations in bacterial attachment (data not shown).

Respiratory burst activity is an oxygen-dependent process related to adequate NADPH-oxidase and mitochondrial activity and is a consequence of attachment and internalization of bacteria (38). We found no significant differences between the three groups in the intracellular hydrogen peroxide formation. However, a similar pattern as in the phagocytosis experiments was observed with a tendency toward an increased hydrogen peroxide formation in the groups expressing increased bacterial adherance. This is consistent with respiratory burst activity as a graded response related to the number of attached and ingested bacteria (39). Our results are similar to unaltered monocyte and neutrophil chemiluminescence in healthy volunteers, reported by Lammi-Keefe *et al.* (40) and Weiner *et al.* (41). In contrast, Fisher *et al.* (7) observed reduced monocyte (7) and neutrophil (8) chemiluminescence in the presence of latex beads and zymosan. The discrepancies may be due to differences in study design.

The aim of the present study was to investigate whether the known antiinflammatory effects of n-3 FA also involve monocyte phagocytosis. For the first time, the separate effects of EPA and DHA were examined. We observed only minor biological changes in monocyte phagocytic functions, indicating that the engulfment process is not affected during n-3 FA supplementation. Retention of phagocytic properties and even improved bacterial attachment after intake of highly purified EPA and DHA may be of importance for the defense against infections to compensate for the known immunosuppressive actions of n-3 FA.

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Failure of the Nonselective β-Blocker Propranolol to Affect Lipoprotein Lipase Gene Expression in the Rat

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ABSTRACT: Treatment with β-blockers has been reported to be associated with the development of hypertriglyceridemia. The etiology, even the existence, of this phenomenon is controversial. The purpose of our study was to examine whether the nonselective β-blocker propranolol causes hypertriglyceridemia in the rat and whether its action is mediated by the modulation of lipoprotein lipase (LPL) messenger RNA (mRNA) accumulation or activity. LPL activity was assayed in fresh tissue by incubation with tritiated triglycerides. LPL mRNA was quantified in total RNA by slot-blot analysis using a mouse LPL complementary DNA probe. We have conducted three series of experiments in unanaesthetized rats in order to study the effects of different single doses of propranolol (1.5 to 6 mg i.p.) and different durations of treatment (15 min to 4 wk). We measured triglyceride and cholesterol levels in plasma as well as the LPL activity and mRNA levels in the heart and adipose tissue before and after propranolol administration. In these experiments we did not find any significant decrease in either the activity or the amount of mRNA of lipoprotein lipase nor was there any change in plasma lipids following treatment. Our results lead us to the conclusion that the nonselective β-blocker propranolol affects neither the activity nor the mRNA level of LPL in the rat. *Lipids 32,* 943–947 (1997).

Lipoprotein lipase (LPL) is the enzyme responsible for the metabolism of lipoproteins rich in triglycerides (TG), i.e., chylomicrons and very low density lipoproteins. Located on the surface of the capillary endothelium, it hydrolyzes TG into glycerol and free fatty acids. In many pathophysiological situations where there is decreased LPL activity, e.g., as in diabetes mellitus, obesity, alcoholism and hypothyroidism, there is concomitant hypertriglyceridemia (1). It is also well known that the use of nonselective β-adrenoceptor blocking agents (β-blockers) may be associated with the development of hypertriglyceridemia (2). The mechanism of this phenomenon is as yet unclear. The purpose of our study was to examine the effect of the acute and chronic administration of the nonselective β-blocker propranolol on LPL activity and LPL messenger RNA (mRNA) in the heart and adipose tissue (the two tissues with the highest amount of LPL) in rats.

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Abbreviations: cRNA, complementary RNA; LPL, lipoprotein lipase; mRNA, messenger RNA; TG, triglycerides.

EXPERIMENTAL PROCEDURES

Chemicals and solutions. [^{3}H]Oleoglycerol (22 mCi/mmol) and Amplify were purchased from Amersham (Arlington Heights, IL). Propranolol, lecithin, triolein, Trasylol, Triton X-100, bovine serum albumin, protein A, EDTA, phenylmethylsulfonylfluoride, HEPES, yeast RNA, β-mercaptoethanol, Krebs-Ringer solution, Leupeptin, and pepstatin were all from Sigma (St. Louis, MO). Heparin sodium salt was from Gibco (Gaithersburg, MD). Zeta-Probe membranes and Tris base were from Biorad (Richmond, CA). GF/B filters were obtained from Whatman (Clifton, NJ). En^{3}hance and Solvable were from Dupont (Wilmington, DE). Human β-actin DNA probe was obtained from Clontech (Palo Alto, CA). Rats were obtained from Harlan (Indianapolis, IN).

Animal procedures. Male Sprague-Dawley rats (approximately 200 g) were maintained under an artificial light–dark cycle of 12 h (lights on at 7:00 a.m.) and were fed rat chow and water *ad libitum*. All animals were studied in the fed state. Propranolol was either administered intraperitoneally (i.p.), with the desired dose suspended in 3 mL of normal saline, or added to the drinking water. Control animals were either injected with 3 mL of normal saline or were given drinking water without propranolol. The rats were sacrificed at various predetermined times after injection. Heart and left epididymal fat pads were excised and frozen immediately in liquid nitrogen. Blood was collected by cardiac puncture for the determination of serum cholesterol and TG.

Serum chemistries. TG and cholesterol levels were determined in serum by using standard Sigma diagnostic kits. Protein was measured by the Coomassie Brilliant Blue method as previously described (3).

LPL assay. LPL activity was assayed using a modification of a previously described technique by Iverius and Östlund-Lindqvist (4). Assays were carried out in a total volume of 500 μL consisting of 300 μL of triacylglycerol emulsion, 75 μL of 0.223 M Tris-HCl (pH 8.5), 25 μL of heat-inactivated (56°C, 30 min) rat serum, and 100 μL of tissue sample. The

reaction was continued at 37°C for 1 h, and 3.25 mL of methanol/chloroform/heptane (1.41:1.25:1.0, by vol) was added to stop the reaction. After vortexing, 1.05 mL of 0.05 M carbonate/borate buffer was added; the reaction mixture was further vortexed and then centrifuged in a benchtop centrifuge at 3,000 × *g* for 15 min. Two mL of the upper aqueous phase were removed, mixed with 4 mL of Aquasol, and radioactivity was measured in a liquid scintillation counter. LPL activity was expressed as nmol of free fatty acids released per mg of protein per hour of incubation.

Extraction of RNA and slot-blot analysis. Isolation of RNA from adipose tissue was performed according to the guanidium–phenol–chloroform method (5), and RNA was quantitated spectrophotometrically. For slot-blot analysis, total RNA was denatured just before blotting. Under our conditions yeast RNA did not show detectable hybridization to the mouse LPL complementary DNA (cDNA). Samples containing approximately 5 µg of total RNA were blotted onto Zeta-Probe membrane using a Minifold II apparatus and fixed by an ultraviolet lamp. A 622 base pair Bam H1 fragment of mouse LPL cDNA (6) was labeled with [^{32}P]dCTP by random oligonucleotide priming. Prehybridization, hybridization, and posthybridization washes were carried out as per the Zeta-Probe protocol. Blots were exposed to film (Kodak XAR-5; Kodak, Ann Arbor, MI) in the presence of two intensifying screens for 20 h at –70°C. Autoradiograms were scanned using a Visage 110 densitometer (BioImage, Kodak). Human β-actin cDNA probe was labeled as described above and used for hybridization.

Statistics. All data are expressed as means ± SEM. Statistical differences were determined by using two-tailed Student's *t*-tests or analysis of variance where appropriate. *P*-values less than 0.05 were considered significant.

RESULTS

We conducted three series of experiments in order to examine the effect of propranolol on LPL activity and LPL mRNA levels in hearts and adipose tissue of rats. In the first series, we studied the effects of a single injection of propranolol at various doses on LPL activity and mRNA. Animals were given i.p. injections of 3 mL of normal saline (controls) or propranolol (1.5 mg to 6 mg/rat, corresponding to 7.5 to 30 mg/kg body weight) and were killed 30 min after injection. TG levels and cholesterol did not increase with any of the doses used (Table 1). Heart LPL activity decreased slightly (about 20%) after the administration of 1.5 and 6 mg of propranolol, but the difference did not reach statistical significance. Adipose tissue LPL activity was decreased by 12, 32, and 33% with doses of propranolol of 1.5, 3, and 6 mg, respectively (nonsignificant). LPL mRNA levels of both heart and adipose tissue (Fig. 1) did not change significantly with any of the doses of propranolol given.

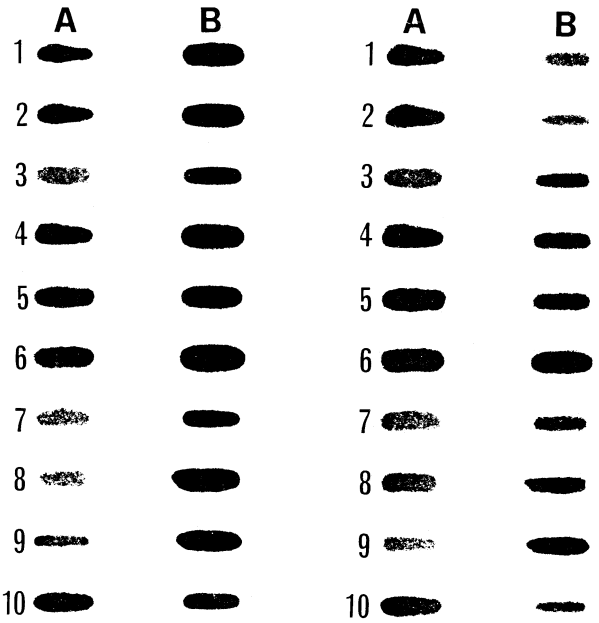

FIG. 1. Autoradiogram of slot-blot analysis of (A) lipoprotein lipase mRNA and (B) β-actin mRNA before (slots #1, 2) and after administration of various doses of propranolol (slots #3, 4 → 1.5 mg; slots 5, 6, 7 → 3 mg; slots 8, 9, 10 → 6 mg); a, adipose tissue and b, heart. 5 µg of total RNA were placed in each slot.

In the second series of experiments, the time course of the effects of propranolol on heart and adipose tissue LPL activities and mRNA was investigated. Animals were given i.p. in-

TABLE 1
Effects of Various Single Doses of Propranolol or Saline (3 mL) Given i.p. 30 min Before Measurements

	Dose of propranolol (mg)			
	0	1.5	3	6
Triglycerides (mg/dL)	95.8 ± 4.6	84.0 ± 4.1	94.0 ± 6.1	82.2 ± 5.6
Cholesterol (mg/dL)	96.5 ± 11.5	88.4 ± 6.2	80.0 ± 18.8	70.0 ± 7.5
Heart LPL activity[a]	32 ± 5	26 ± 5	32 ± 4	26 ± 4
Adipose tissue LPL activity[a]	188 ± 27	166 ± 19	128 ± 12	126 ± 11
Heart LPL mRNA[b]	5.4 ± 1.67	2.6 ± 0.78	5.8 ± 1.42	3.8 ± 1.0
Adipose tissue LPL mRNA[b]	1.45 ± 0.53	1.22 ± 0.2	1.46 ± 0.12	2.05 ± 0.25

[a]Expressed as nmol of free fatty acids released per mg of protein per hour of incubation. Data are means ± SEM of $n = 5$ animals per group.
[b]Expressed as ratio lipoprotein lipase (LPL) mRNA/β-actin mRNA.

TABLE 2
Time Course of the Effects of Single Doses (3 mg i.p.) of Propranolol or Saline (3 mL)

	Time after injection (min)				
	0	15	30	60	120
Triglycerides (mg/dL)	112 ± 13	122 ± 11	116 ± 10	107 ± 11	133 ± 6
Cholesterol (mg/dL)	63 ± 1.9	61 ± 6.8	70 ± 11	74 ± 10	65 ± 6.1
Heart LPL activity[a]	22 ± 4	21 ± 4	26 ± 3	26 ± 2	20 ± 2
Adipose tissue LPL activity[a]	98 ± 19	153 ± 23	57 ± 11	120 ± 15	176 ± 39
Heart LPL mRNA[b]	1.22 ± 0.31	0.92 ± 0.07	0.93 ± 0.02	1.14 ± 0.07	1.37 ± 0.04
Adipose tissue LPL mRNA[b]	2.06 ± 0.53	2.82 ± 0.68	1.4 ± 0.07	2.75 ± 0.38	2.99 ± 0.04

[a]Expressed as nmol of free fatty acids released per mg of protein per hour of incubation. The animals were killed at various time points after injection. Data are means ± SEM of $n = 5$ animals per group.
[b]Expressed as ratio LPL mRNA/β-actin mRNA. See Table 1 for abbreviation.

jections of 3 mg of propranolol and were killed at 15, 30, 60, and 120 min after the injection. TG and cholesterol concentrations remained unchanged (Table 2). The observed fluctuations in the rat heart and adipose tissue LPL activities were not statistically significant. Heart and adipose tissue LPL mRNA did not change significantly at any point during the treatment period.

In the third series of experiments, we studied the effects of chronic propranolol administration on serum TG and cholesterol levels, on LPL activity, and LPL mRNA in rat heart and adipose tissue in order to investigate any possible influence of propranolol on LPL during long-term administration. In two separate experiments, we added propranolol to the drinking water of the rats for 2 and 4 wk, respectively. Each rat received 3 mg/day of propranolol, and the water was renewed every other day. After 2 wk of propranolol administration, there was no statistically significant difference in the TG and cholesterol concentrations (Table 3). Heart LPL activity decreased by 20%, a statistically nonsignificant change and there was no change in the adipose tissue LPL activity. Both heart and adipose tissue LPL mRNA did not change. After 4 wk of propranolol administration, the TG and cholesterol levels did not change significantly (Table 3). Furthermore, there were no statistically significant changes in heart and adipose LPL activities. Heart LPL mRNA increased by 6%, from 0.816 ± 0.03 to 0.864 ± 0.077. Adipose tissue LPL mRNA increased by 7%, from 0.894 ± 0.0193 to 0.9611 ± 0.027. Both changes were not statistically significant.

DISCUSSION

In the present study, we investigated the effects of the nonselective β-blocker propranolol on the expression of the lipoprotein lipase gene. We measured the TG and cholesterol levels in blood, the LPL activity, and the amount of LPL mRNA with and without propranolol administration. There was no significant influence of the drug on any of the parameters determined after single injections of propranolol or during chronic oral administration of the drug for up to 4 wk.

In the literature, there are many contradictory reports on the effects of nonselective β-blockers on blood lipids. Some authors have found that β-blockers are associated with hypertriglyceridemia (2,7–10). Others failed to find such an association (11–13). Most of the aforementioned studies were done in humans with the exception of those of Jansen and Baggen (12) and Dall'Aglio *et al.* (13), which were done in rats.

The effect of nonselective β-blockers on serum cholesterol was also controversial (10,12). Catecholamines were found to decrease the number of LDL receptors and therefore increase the cholesterol levels in blood, since the latter are inversely related to the number of LDL receptors. *In vitro* studies have shown that catecholamines also inhibit cholesterol synthesis in extrahepatic cells through their action on α_2 and β_2 receptors (14). Propranolol therefore could act by suppressing the catecholamine actions (15), but catecholamines themselves do not seem to exert any major influence on lipid metabolism (16). Therefore, it is unlikely that the increase in

TABLE 3
Effects of Chronic Administration of Propranolol (3 mg per day)[a]

	Water only (2 wk)	Propranolol (2 wk)	Water only (4 wk)	Propranolol (4 wk)
Triglycerides (mg/dL)	147.5 ± 6.9	140.0 ± 7.0	114.6 ± 7.4	105.9 ± 1.9
Cholesterol (mg/dL)	70.9 ± 10.7	70.9 ± 6.5	81 ± 4.9	78 ± 2.6
Heart LPL activity[b]	31 ± 1	25 ± 2	10 ± 2	9 ± 1
Adipose tissue LPL activity[b]	147 ± 18	125 ± 32	131 ± 47	116 ± 17
Heart LPL mRNA[c]	1.0 ± 0.05	0.94 ± 0.06	0.82 ± 0.03	0.86 ± 0.08
Adipose tissue LPL mRNA[c]	1.39 ± 0.01	1.26 ± 0.06	0.89 ± 0.02	0.96 ± 0.03

[a]For 2 or 4 wk with the drinking water. Data are means ± SEM of $n = 20$ animals per group.
[b]Expressed as nmol of free fatty acids released per mg of protein per hour of incubation.
[c]Expressed as ratio LPL mRNA/β-actin mRNA. See Table 1 for abbreviation.

plasma catecholamine levels after β-blocker treatment will affect lipid levels.

Contradictory results also were reported in the literature on the effect of β-blockers on LPL activity. Some authors found an increase in the activity of the enzyme (17) while others (11) found a decrease. We could not document any changes in LPL activity after acute or chronic administration, and our results are in agreement with those of other authors (12,18). These contradictory results may be due to the use of different laboratory methods of LPL activity measurements, different tissues in which LPL activity was measured (heart, adipose, serum), use of different β-blockers, as well as variation in doses and duration of treatment. Another factor is that lipoprotein levels as well as LPL activity change during the day and at different seasons. It is also possible that rats and humans respond differently to the administration of β-blockers. Nonetheless most researchers believe that rats are a good model for the study of the effects of the adrenergic system on lipid metabolism (12,13).

In our study, we administered propranolol to rats for up to 4 wk with no significant change in the serum TG levels. This can be due to the fact that, in order for hypertriglyceridemia to develop, an even longer time of treatment is required. Some authors (19) observed an increase in TG levels only after 1 yr of treatment, others (11) after 2 mon and others (8) after 3 mon. There is also the possibility that in order for hypertriglyceridemia to develop there needs to be an underlying lipid abnormality. It has been shown that patients who already had hypertriglyceridemia and were treated with propranolol showed an increased lipemic response to a meal high in fat compared to patients with normal initial TG levels treated with propranolol (20).

Despite the lack of effects of propranolol on serum lipoproteins in the present experiments, an effect on LPL activity or mRNA expression could not be excluded. There is a body of evidence showing that catecholamines affect LPL activity. Stimulation of β-receptors activates LPL (10), so their blockade could result in the opposite effect, i.e., LPL suppression. It also has been shown that factors that increase cAMP (through which catecholamines exert their actions), like β-blockers and the cholera toxin, also increase muscle LPL activity (21). Furthermore it is known that adrenaline decreases LPL synthesis and increases the rate of LPL catabolism in rat adipose tissue (22). Evidence also suggests that adrenaline affects LPL at a posttranslational level, changing the enzyme's secretion and activation (23). It has been observed that adrenaline decreases LPL activity in rat heart and in isolated cardiac myocytes (24). Others have observed that cAMP increases LPL synthesis within 2 h in isolated rat heart cells (25). Furthermore, catecholamines, cAMP and isoproterenol, acting through β-receptors, suppress LPL activity in white adipose tissue of rats *in vitro* (26), in heart and skeletal muscles (27), and in brown adipose tissue (28).

In summary, the results of our study support the conclusion that, under the conditions used, the nonselective β-blocker propranolol does not affect LPL activity and gene expression in the rat.

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Effects of Triolein or Oleic Acid on Lymphatic Recovery of Docosahexaenoic Acid Given as Ethyl Ester and Their Intramolecular Distribution in Lymph Triglyceride of Rats

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ABSTRACT: Effects of oleic acid or triolein on lymphatic recovery of docosahexaenoic acid (DHA) given as an ethyl ester were examined in rats with cannulated thoracic ducts. Lymphatic recovery of ethyl DHA given with oleic acid or triolein was significantly higher than in rats given ethyl DHA alone. DHA distributed almost exclusively at the 1- and 3-position of triglyceride in lymph collected at 0–3 h after the administration, when it was given with oleic acid or triolein. A small part of DHA distributed at the 2-position when ethyl DHA was the sole fatty acid given. Oleic acid given as free acid or triolein with ethyl DHA was a major fatty acid at the 2-position. Intramolecular distribution of DHA and oleic acid in lymph triglyceride was similar when ethyl DHA was given with oleic acid or triolein.

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It has been reported that docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids in marine oils have beneficial physiological functions in experimental animals and humans (1,2). Different functions between DHA and EPA also have been reported (3). Ethyl esters of DHA and EPA are utilized as a nutritional supplement and a therapeutic. We and other investigators observed that intestinal absorption of DHA and EPA given as ethyl ester was lower than seen in the case of triglyceride or free acid (4–8). The low absorbability of the ethyl esters was ascribed to the low hydrolysis rate by pancreatic lipase (5,9). However, there were observations that when EPA and DHA were given as ethyl ester or as triglyceride to humans, increases of these n-3 polyunsaturated fatty acids in plasma were comparable (10,11).

After incorporation into intestinal cells, almost all fatty acids are synthesized to triglyceride. The intramolecular distribution of EPA and DHA in lymph triglyceride is scanty when these are given as an ethyl ester (12). This information may be important because the difference in intramolecular distribution of EPA and DHA in lymph triglyceride may influence related physiological functions (13,14).

Effects of coexisting free oleic acid or triolein on recovery of DHA given as ethyl ester were examined in this study in rats with cannulated thoracic ducts, and the intramolecular distribution of DHA and oleic acid in chylomicron triglyceride was measured.

MATERIALS AND METHODS

DHA ethyl ester (purity >97%) was kindly provided by Dr. K. Yazawa, Sagami Chemical Research Center, Sagamihara, Kanagawa, Japan. Triolein (purity 99%) and oleic acid (purity 99%) were obtained from Sigma Chemical Co. (St. Louis, MO). Fatty acid-free bovine serum albumin was obtained from Miles Scientific, ICN ImmunoBiologicals (Lisle, IL). Sodium taurocholate (purity >98%) was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Male Sprague-Dawley rats (Seiwa Experimental Animals, Fukuoka, Japan) weighing 310–330 g were provided *ad libitum* a commercial nonpurified diet (type NMF; Oriental Yeast Co., Tokyo) and tap water for drinking. Thoracic duct was cannulated, and an indwelling catheter was placed in the stomach (4,5). After this surgery, the rats were placed in restraining cages in a warm recovery room. They were given a normal osmotic solution containing 139 mM glucose and 85 mM NaCl as drinking water, which was infused at a rate of 3 mL/h *via* the gastric tube until the end of the experiment. The morning after collection of lymph for 2 h (blank lymph), each animal was given 3 mL of a test emulsion through the gastric tube, and the lymph was collected for 24 h beginning at 10:00 a.m. The 3-mL test emulsion contained 200 mg of sodium taurocholate, 50 mg of fatty acid-free albumin, and either 100 or 200 mg of ethyl DHA, 100 mg of ethyl DHA plus 100 mg of oleic acid, or 100 mg of ethyl DHA plus 100 mg of triolein. Six rats were in each group.

Lymph lipid extracted with 20 vol of chloroform/methanol (2:1, vol/vol) (15) was transmethylated with a BF_3–methanol complex. Fatty acid methyl esters were subjected to gas–liquid chromatography on a 10% Silar 10C (Chromato TEC, Osaka, Japan) column (4,5). Pentadecanoic acid (Aldrich Chemical Co., Milwaukee, WI) was used as an internal stan-

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

dard. The fatty acid recovery rate was calculated by subtracting the fatty acid amount in the blank lymph from that in the lymph collected after the administration of a test emulsion.

Lymph collected at 0–3 h was subjected to determination of the triglyceride structure. After separation of triglyceride by silica gel G thin-layer chromatography developed with petroleum ether/diethyl ether/acetic acid (82:18:1, by vol), triglyceride was extracted with diethyl ether. Intramolecular distribution of triglyceride was analyzed by a Grignard degradation method (14). To triglyceride (about 10 mg) dissolved in 0.6 mL of diethyl ether was added 0.25 mL of 1 M ethylmagnesium bromide in diethyl ether (Aldrich Chemical Co.) under continuous mixing with a magnetic stirrer. After 25 s, 0.25 mL of acetic acid/diethyl ether (1:1, vol/vol) was added to stop the reaction, and 5 mL of 10% boric acid solution was added while mixing. The reaction mixture was extracted using 5 mL of diethyl ether saturated with boric acid. The diethyl ether phase was washed twice with 1 mL of 2% aqueous $NaHCO_3$ and twice with 1 mL water. The solution was dried using a small amount of anhydrous sodium sulfate and nitrogen at room temperature. The lipids were applied to a thin-layer chromatography plate containing 5% boric acid, and the plate was developed with chloroform/acetone (96:4, vol/vol). 2-Monoglyceride and 1,3-diglyceride fractions were transmethylated, and the fatty acid methyl ester was analyzed with gas–liquid chromatography, as described above.

Data are expressed as means ± SEM. Duncan's new multiple-range test was used to determine the exact nature of the difference among the groups (16).

RESULTS

Lymph flow rate was linear, and there was no significant difference among the groups (Fig. 1). The lymph flow rate was sufficient to the extent that lipid absorption was not interfered with (5,17).

Lymphatic recovery of oleic acid is shown in Figure 2. Lymphatic recoveries of oleic acid given as ethyl DHA + oleic acid and ethyl DHA + triolein were quantitative for 24 h (100 ± 4 and 104 ± 2%, respectively), as observed under similar experimental conditions (5,17). When ethyl DHA 100 and 200 mg was given, lymphatic recoveries of DHA for 24 h were 69.9 ± 1.8% and 64.0 ± 3.3%, respectively (Fig. 3), with a significant difference, at $P < 0.05$. The addition of oleic acid or triolein significantly increased the recovery of DHA in lymph compared with findings in groups given ethyl DHA alone. The accelerated recoveries of DHA were mainly observed in lymph at 0–3 h, and the recoveries after 3 to 24 h were comparable among the four groups. The 24-h recoveries of DHA were 84.4 ± 3.0 and 84.8 ± 2.3% in rats given ethyl DHA + oleic acid and ethyl DHA + triolein, respectively. In our previous study done under similar experimental conditions, lymphatic recoveries for 24 h of DHA given as free acid and triglyceride were 82.7 and 78.9%, respectively (5). Therefore, these recovery rates of DHA were thought to be maximal, at least in our setup.

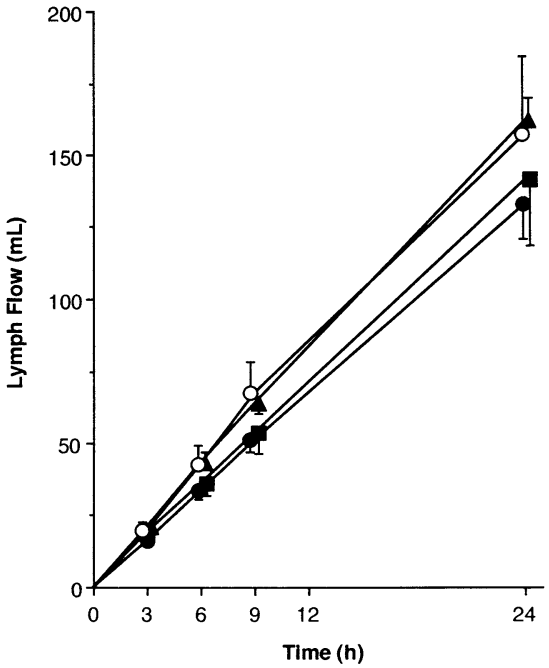

FIG. 1. Lymph flow rate. ●; 200 mg of ethyl docosahexaenoic acid (DHA); ○, 100 mg of ethyl DHA; ■, 100 mg of ethyl DHA + 100 mg of oleic acid; ▲, 100 mg of ethyl DHA + 100 mg of triolein. Data are mean ± SE of six rats.

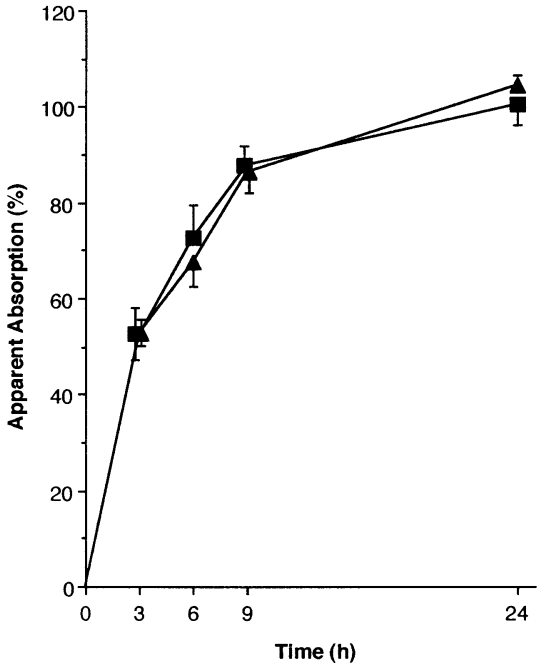

FIG. 2. Lymphatic recovery of oleic acid in the thoracic duct lymph of rats after intragastric administration of a fat emulsion containing ethyl DHA + oleic acid or ethyl DHA + triolein. ■, ▲, oleic acid recovery given as 100 mg of ethyl DHA + 100 mg of oleic acid and 100 mg of ethyl DHA + 100 mg of triolein, respectively. Data are mean ± SE of six rats. See Figure 1 for abbreviation.

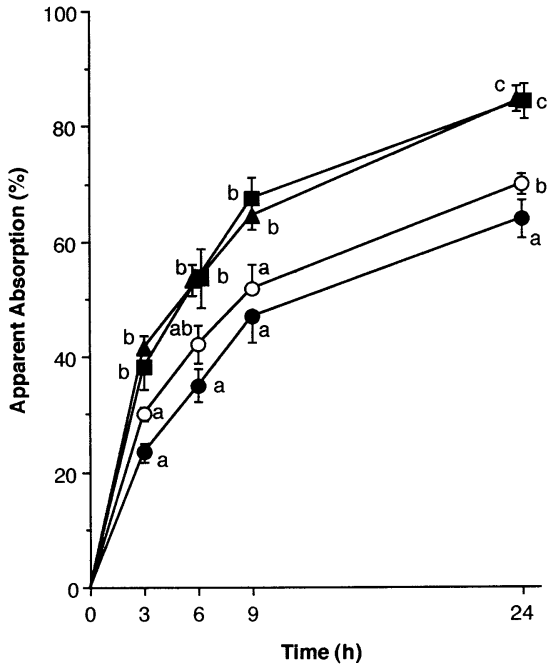

FIG. 3. Lymphatic recovery of DHA in the thoracic duct lymph of rats after intragastric administration of a fat emulsion containing ethyl DHA, ethyl DHA + oleic acid, or ethyl DHA + triolein. ●, 200 mg of ethyl DHA; ○; 100 mg of ethyl DHA; ■, 100 mg of ethyl DHA + 100 mg of oleic acid; and ▲, 100 mg of ethyl DHA + 100 mg of triolein, respectively. Data are mean ± SE of six rats. Values with different letters among the DHA recoveries are significantly different at $P < 0.05$. See Figure 1 for abbreviation.

When ethyl DHA was given as a sole fat, DHA was mainly incorporated into the 1- and 3-position of triglyceride in lymph collected at 0–3 h after administration (Table 1). DHA (84%) in lymph triglyceride was located at 1- and 3-position, whereas 16% of DHA was incorporated into the 2-position. The endogenous fatty acids mainly occupied the 2-position. When oleic acid or triolein was simultaneously given with ethyl DHA, DHA was incorporated almost exclusively at the 1- and 3-position (99% of DHA in lymph triglyceride in both groups). In contrast, a major fatty acid in the 2-position was oleic acid; 46 and 49% of oleic acid given as ethyl DHA + oleic acid and ethyl DHA + triolein, respectively, were estimated to be located at the 2-position, and the remaining parts were at the 1- and 3-position. No difference on the intramolecular distribution of DHA and oleic acid was observed between these two groups.

DISCUSSION

Under the coexistence of triolein or oleic acid, lymphatic recoveries of DHA given as ethyl ester increased to a level almost comparable to cases of free fatty acid and triglyceride, as previously reported (5). Hydrolysis products by pancreatic lipase of triglyceride, 2-monoglyceride and free fatty acid, are surface-active and accelerate emulsification of lipids (18). Therefore, it is thought that oleic acid and 2-monoolein increase the accessibility of ethyl DHA to pancreatic lipase and hence increase the rate of hydrolysis. The accelerated recovery of DHA observed in lymph only at 0–3 h (Fig. 3) again suggests that ethyl DHA is rapidly hydrolyzed and absorbed when given with oleic acid or triolein. Although one study did suggest that EPA given as ethyl ester was absorbed at the same rate as administered triglyceride (19), several investigations showed that lymphatic recovery of EPA and DHA, given as ethyl ester, was lower than when given to rats in the triglyceride form (4,5,7). Ethyl ester was given solely in these studies. Therefore, there is the possibility that the rate of hydrolysis of ethyl ester by pancreatic lipase is low under these experimental conditions. In this context, Lawson and Hughes (20) reported that absorption of EPA and DHA from ethyl esters of fish oil fatty acids was increased by coingestion with a high-fat meal compared to low-fat meal in humans. Our results indicate that utilization of ethyl DHA does not reduce the availability as a DHA supplement, if it is ingested with a meal containing dietary fat.

Since the 2-monoglyceride pathway is more active than the phosphatidic acid pathway in intestinal cells (21), there is the possibility that the existence of 2-monoolein formed by hydrolysis of triolein accelerates triglyceride synthesis and secretion into lymph. In our previous study, lymphatic recoveries of DHA and EPA given as free fatty acids were delayed compared with findings when the triglyceride form was given (5). Yang *et al.* (22) also suggested that low absorbability of the ethyl ester may be due to lack of 2-monoglyceride on resynthesis of triglyceride in intestinal cells. However, in the present study oleic acid and triolein had almost the same effect on lymphatic recovery of DHA. Thus, the lack of 2-

TABLE 1
Intramolecular Distribution (mol%) of Fatty Acids in Rat Lymph Triglyceride[a]

	Ethyl DHA[b]			Ethyl DHA + oleic acid			Ethyl DHA + triolein		
Fatty acids	Total	*sn*-2	*sn*-1,3	Total	*sn*-2	*sn*-1,3	Total	*sn*-2	*sn*-1,3
16:0	7.5 ± 1.2	14.5 ± 2.4	4.0 ± 2.9	5.2 ± 0.4	6.6 ± 0.5	4.5 ± 0.4	4.6 ± 0.4	5.6 ± 1.2	4.1 ± 1.2
18:0	4.3 ± 0.3	3.1 ± 0.2	5.0 ± 0.4	2.6 ± 0.1	1.2 ± 0.1	3.3 ± 0.2	3.4 ± 0.1	2.4 ± 0.4	3.9 ± 0.2
18:1	4.7 ± 0.5	8.9 ± 0.8	2.6 ± 0.5	55.3 ± 1.0	76.6 ± 2.1	44.7 ± 1.2	50.5 ± 1.1	73.5 ± 0.9	38.9 ± 1.5
18:2n-6	12.1 ± 0.8	31.7 ± 1.6	2.3 ± 0.5	7.7 ± 0.4	12.4 ± 0.8	5.4 ± 0.4	8.9 ± 0.1	15.2 ± 0.5	5.8 ± 0.2
20:4n-6	3.4 ± 0.2	8.5 ± 0.5	0.8 ± 0.1	1.8 ± 0.2	3.1 ± 0.3	1.2 ± 0.1	1.5 ± 0.1	2.2 ± 0.2	1.1 ± 0.2
22:6n-3	66.5 ± 2.5	31.5 ± 1.7	84.0 ± 3.9	27.3 ± 0.9	0.2 ± 1.6	40.9 ± 1.4	31.2 ± 1.4	1.2 ± 2.2	46.2 ± 2.7

[a]0–3 h after gavage administration. Data are mean ± SE of six rats. Lymph was subjected to determination of intramolecular structures of triglyceride.
[b]Lymph collected from rats given 200 mg ethyl docosahexaenoic acid (DHA).

monoglyceride is not rate-limiting under these experimental conditions.

Intramolecular distribution of DHA in lymph triglyceride was similar when given with oleic acid or triolein, and DHA was specifically distributed to the 1- and 3-position (Table 1). The result is consistent with the report in which EPA and DHA were mainly distributed at the 1- and 3-position in lymph chylomicron triglycerides of rats given menhaden oil fatty acids as ethyl ester (12). These results suggest that DHA is specifically bound to the 1- and 3-position in triglyceride synthesis *via* the phosphatidic acid pathway. When ethyl DHA was given as a sole fat, a small portion of DHA was incorporated into the 2-position of triglyceride in lymph collected at 0–3 h after administration. Fatty acid absorption and triglyceride synthesis are active in intestinal epithelial cells during this time. There is the possibility that a supply of endogenous fatty acids bound to the 2-position of triglyceride is insufficient during the period when DHA is actively absorbed. Therefore, although DHA has a low specificity at the 2-position, part of DHA might be incorporated into the 2-position. Since little information is available on fatty acid specificity in triglyceride synthesis *via* the phosphatidic acid pathway, more detailed studies are necessary.

We reported that DHA and EPA distributed at the 1- and 3-position of triglyceride were conserved in chylomicron triglyceride and these acids had stronger triglyceride-lowering activity in plasma and liver compared to the case of distribution at the 2-position (14). Although little is known of the influence of fatty acids other than oleic acid on intramolecular distribution of DHA in triglyceride, there is the possibility that DHA given as ethyl ester has physiological functions similar to triglycerides in which EPA and DHA are distributed at the 1-, and 3-position.

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The Cholesterol-Lowering Effect of Guar Gum Is Not the Result of a Simple Diversion of Bile Acids Toward Fecal Excretion

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ABSTRACT: The effects of partially hydrolyzed, nonviscous, guar gum (PHGG) on cholesterol metabolism and digestive balance have been compared with those of native guar gum (GUAR) in rats adapted to 0.4% cholesterol diets. Both types of guar gum elicited acidic fermentations in the large intestine, but only GUAR effectively lowered plasma cholesterol ($P < 0.001$), chiefly in the triglyceride-rich lipoprotein fraction. The biliary bile acid excretion was significantly enhanced in rats fed GUAR ($P < 0.05$), as well as the intestinal and cecal bile acid pool ($P < 0.001$). In rats fed GUAR and to a lesser extent in those fed PHGG, the fecal excretion of bile acids and neutral sterol was higher than in controls ($P < 0.01$). The digestive balance (cholesterol intake–steroid excretion) was positive in control rats (+47 μmol/d), whereas it was negative in rats fed GUAR (−20 μmol/d), which could involve a higher rate of endogenous cholesterol synthesis. In rats fed PHGG, the steroid balance remained slightly positive. Liver 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity was very low (22 pmol/min/mg protein), owing to cholesterol supplementation, in control rats or in rats fed PHGG, whereas it was markedly higher (+463%) in rats fed GUAR. In conclusion, even if PHGG does alter some parameters of the enterohepatic cycle of cholesterol and bile acids, its effects are not sufficient to elicit a significant cholesterol-lowering effect. The intestinal (ileal or cecal) reabsorption of bile acids was not reduced, but rather increased, by GUAR; nevertheless the intestinal capacities of reabsorption were overwhelmed by the enlargement of the digestive pool of bile acids. In the present model, induction of HMG-CoA reductase probably takes place in the presence of elevated portal bile acid concentrations.

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Some water-soluble dietary fibers, such as guar gum, have been shown to be hypocholesterolemic in humans or in experimental animals (1,2). Their effectiveness on cholesterolemia has generally been connected to the high viscosity of guar gum. Viscous hydrocolloids may reduce the rate of gastric emptying and the diffusion of nutrients to the mucosa of the small intestine. These mechanisms reduce the absorption of cholesterol, whatever its origin (dietary or endogenous). Steroid sequestrants (cholestyramine or cyclodextrin) are considered as effective as cholesterol-lowering agents (3), but recent investigations suggest that readily fermented substrates, such as resistant starch, may also be very effective in this respect (4). In fact, emphasis has been put on the capacity of fibers to entrap bile acids in the small intestine and to increase their fecal excretion: this should accelerate cholesterol oxidation to bile acids, resulting in a spillover of the body cholesterol pool, due to fecal losses of steroids. It has been proposed that guar gum inhibits the reabsorption of bile acids, especially in the ileum where system(s) of facilitated transport of bile acids are present (5–7). Furthermore, soluble fibers such as guar gum are almost completely fermented by hind-gut microflora to short-chain fatty acids (SCFA), and this process is characterized by an acidification of the cecal content and by a rise in the bacterial mass. These changes are liable to promote bile acid insolubilization and/or trapping of solids, hence a reduced passive reabsorption in the large intestine.

One major drawback of guar gum is its very high viscosity, which alters the acceptability and the palatability of the food in which it is included (8). Thus, attempts have been made to prepare less viscous forms of guar gum, generally by partial enzymatic hydrolysis. However, when guar gum is hydrolyzed with a subsequent reduction of its viscosity, its efficacy may be greatly diminished (9). Yet Ide *et al.* (10) have reported that partially hydrolyzed guar gum may affect biliary bile acid secretion and lead to a cholesterol-lowering effect in rats fed high-fat diets.

The present study was carried out in rats adapted to 0.4% cholesterol diet to compare the lipid-lowering effectiveness of a high-viscosity guar gum (GUAR) or a low-viscosity, partially hydrolyzed guar gum (PHGG) and to examine the impact of these guar gum preparations on bile acid secretion, reabsorption, and excretion and the consequences on the steroid balance in the digestive tract. We also examined whether the hypothe-

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Abbreviations: HDL, high density lipoprotein; PHGG, partially hydrolyzed guar gum; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; GUAR, natural guar gum; SCFA, short-chain fatty acid.

sis of an impaired reabsorption of bile acids with diets containing guar gum is valid and whether other processes could play a role in the cholesterol-lowering effect of guar gum.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats (IFFA-CREDO, l'Arbresle, France) weighing approximately 150 g were fed for 21 d with semipurified diets distributed as a moistened powder. The control diet contained the following (in g/kg diet): casein (L. François, Paris, France) 150; corn oil 50; cholesterol 4; wheat starch 726; mineral and vitamin mixes (Usine d'Alimentation Rationnelle, Villemoisson/Orge, France) 60 and 10, respectively. The diet of the two experimental groups contained 80 g guar gum/kg diet, at the expense of wheat starch. Guar gum flours were purchased from Meyhall (Kreuzlingen, Switzerland). Two guar gum flours with different molecular weights (MW) and particle sizes were studied: a native guar gum (GUAR; Meyproguar) of high MW ($\approx 2 \cdot 10^6$) and a PHGG (Fiberon) which was produced by β1,4-mannanase (E.C. 3.3.1.78). The hydrolysate of PHGG was then separated, purified, dried, and powdered to produce a product of low MW (≈15,000). The viscosity of PHGG in distilled water was not higher than 10 cP, even at 10% concentration, which is much less viscous than the starting material GUAR, which showed a viscosity of 2800–3400 cP at 1% concentration. Animals were housed two per cage and maintained in temperature-controlled rooms (22°C), with the dark period from 2000 to 0800 h. Rats were maintained and handled according to the recommendations of the Institutional Ethics Commitee (Clermont-Ferrand University). The body weight of rats was recorded every 48 h during the experimental period; food intake and fecal excretion were recorded over three 2-d periods throughout the last 10 d.

Sampling procedures. Rats were killed at the end of dark period, namely at a time at which cecal fermentations are still very active. They were anesthetized with sodium pentobarbital (40 mg/kg) and maintained at 37°C. An abdominal incision was made, and blood (1 mL) was withdrawn from both the portal vein and from the abdominal aorta. For blood flow measurement, bromosulfophthalein in saline (5 mmol/L) was infused at a rate of 50 µL/min into the small afferent vein on the internal curvature of the cecum: dilution of the marker in the vein draining the whole cecum allows determination of the cecal blood flow. The blood of each animal was placed in a plastic tube containing heparin and centrifuged at $10,000 \times g$ for 5 min. After centrifugation, plasma was removed and kept at +4°C for lipid and lipoprotein analysis. After blood sampling, the cecum with contents was removed and weighed, two samples of cecal content were transferred to microcentrifuge tubes and immediately frozen at –20°C.

The small intestine was clamped at the pylorus and the ileal-cecal junction, removed, stripped of mesentery and fat, and weighed. The small intestine was halved to facilitate handling, and the content of each section was emptied into a preweighed tube by finger stripping then frozen at –20°C.

Surgical procedure. In a separate series of anesthetized rats, a mid-line laparotomy was performed and the bile duct exposed and ligated distally. The bile duct was then catheterized with a PE10 polyethylene catheter (Biotrol, Paris, France), and bile was allowed to drain for 30 min and then collected for two 30-min periods into preweighed vials cooled on ice. Bile volume was determined gravimetrically.

Analytical procedures. SCFA concentrations were measured by gas–liquid chromatography (GLC) after ethanolic extraction of plasma samples as described by Rémésy and Demigné (11) and on supernatants ($8,000 \times g$, 5 min at 4°C) of cecal contents. Bile acids analysis was effected either directly on plasma and bile or on extracts from cecal content or small intestine and feces using 10 vol ethanolic KOH (0.5 M); analyses were quantified using the reaction catalyzed by the 3α-hydroxysteroid dehydrogenase (E.C. 1.1.1.50; Sigma, L'isle d'abeau, Chesnes, France). The soluble bile acids levels were determined on supernatants of cecal contents by the enzymatic method described above. Neutral steroids were extracted three times with 1 mL hexane from a 100-µL aliquot of the alkaline ethanolic extract; after addition of 5α-cholestane as an internal standard, the solvent was then evaporated under a stream of N_2 and the residue dissolved in hexane. Portions (0.5 µL) of this extract were injected into a gas chromatograph (Delsi 330, Paris, France) equipped with a 12 m × 0.25 mm i,d. fused-silica capillary column (BP10; SGE, Villeneuve-St-Georges, France) and a flame-ionization detector. Helium was used as a carrier gas, and the sterols were separated isothermally at 260°C. Sterols were calculated from the peak areas relative to the peak area of the internal standard. Triglycerides (Biotrol) and total cholesterol (BioMérieux, Charbonnières-les-Bains, France) were determined in plasma and bile (only cholesterol) by enzymatic procedures.

Plasma lipoproteins were separated on a density gradient by preparative ultracentrifugation (12) in a TST 41.14 swinging-bucket rotor (Kontron, Zürich, Switzerland) at $100,000 \times g$ for 36 h (15°C). Because of the low level of plasma low-density lipoprotein and the partial overlapping of high density lipoprotein 1 (HDL1) and HDL2 fractions in rats, it was considered only two fractions: the fractions of $d < 1.040$ kg/L (chiefly triglyceride-rich lipoprotein, together with some low density lipoprotein) and the fraction of $d > 1.040$ kg/L (HDL). The cholesterol contents of each fraction were determined by the method described above.

Liver triglyceride and cholesterol were extracted and analyzed as described by Mazur *et al*. (13), and a control serum (Biotrol-33 Plus, Biotrol) was treated in parallel to samples and served as a control of accuracy of analysis.

Enzyme activities. Two grams of liver were homogenized in 4 mL of an ice-cold buffer 1 (50 mmol/L, Tris hydrochloride; 250 mmol/L, sucrose; 50 mmol/L, EDTA; 2 mmol/L, dithiothreitol; and 2 µmol/L, leupeptin, pH 7.2) with a Potter-Elvejhem homogenizer (Braun, Melsungen, Germany) at moderate speed. The homogenate was first centrifuged at $10,000 \times g$ (15 min, 4°C); the resulting supernatant was then

centrifuged at 100,000 × *g* (60 min, 4°C). Pellets were resuspended in 2 mL of chilled buffer 1. The centrifugation procedure was repeated and the resulting pellets homogenized in 1 mL of buffer 2 (sucrose, 100 mmol/L; KCL, 50 mmol/L; K phosphate, 40 mmol/L; EDTA, 30 mmol/L; dithiothreitol, 1 mmol/L; pH 7.2). The microsomal preparation was stored at −80°C until measurement of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity. The protein content of microsomes was determined using the Pierce BCA reagent kit (Interchim, Montluçon, France). The activity of HMG-CoA reductase (E.C. 1.1.1.34) was determined on microsomal fractions as described by Wilce and Kroone (14). Labeled mevanololactone was separated from unreacted HMG-CoA by column chromatography using AG1-X8 resin (200 to 400 mesh, formate form; BioRad, Paris, France). Specific radioactivity of the enzyme was expressed in picomoles of [3-^{14}C]HMG-CoA transformed in [^{14}C]mevalonolactone per minute per microgram of microsomal protein, after correcting for recovery of [^{3}H]mevalonolactone from the column.

A portion of liver was freezed-clamped and stored at −80°C for the measurement of fatty acid synthase activity and liver lipids. The cytosolic extract was prepared by treating a 100-mg aliquot with 3 mL isotonic phosphate buffer (K phosphate, 143 mM; 2-mercaptoethanol, 5 mM; EDTA, 4 mM; pH 6.8) using a Polytron homogenizer (Kinematica AG, Littau, Switzerland), then centrifuged at 48,000 × *g* (30 min at 4°C). The enzyme activity of fatty acid synthase was determined by an isotopic method: [2-^{14}C] malonyl CoA was used as a tracer (15). Fatty acid synthase activity was expressed in nanomoles of [2-^{14}C]malonyl CoA incorporated into fatty acids per minute per gram of liver.

Calculation and data analysis. The small intestinal and cecal pool was calculated as: concentration (mmol/L) × digestive volume (mL). Cecal bile acids absorption was determined using arteriovenous difference and blood flow measurement ([cecal vein] − [artery]) × cecal blood flow. The apparent neutral sterol absorption was calculated as: cholesterol intake (≈245 µmol/d) − fecal excretion of neutral sterols. The apparent cholesterol digestibility was calculated as: apparent neutral sterol absorption (µmol/d)/cholesterol intake (µmol/d) × 100, and the total steroids retention as: cholesterol intake − (fecal excretion of neutral sterols + fecal excretion of bile acids). Values are given as the means ± SEM and, where appropriate, significance of differences ($P < 0.05$) between mean values was determined by analysis of variance coupled with the Student's Newman-Keuls' Test.

RESULTS

Effects of diets on body weight, cecal development, cecal pH, and cecal fermentations. The data presented in Table 1 show that there were no significant differences in body weight between control rats and rats fed the guar gum diets. The cecum weight was significantly enhanced in rats fed GUAR and PHGG diets (+73% and +65%, respectively), with a parallel increase of cecal wall weight. Diets containing GUAR or PHGG caused an acidification of the cecal content (pH 5.95 and 6.01, respectively, compared to 7.22 for control rats). Rats fed the GUAR or PHGG diets had cecal SCFA concentrations in the range of 140–160 mmol/L, compared to 79 mmol/L for controls. In parallel, the total SCFA pool was significantly enhanced in rats fed the GUAR or PHGG diets (≈+250% compared to controls); furthermore, there was a higher molar proportion of propionate in rats fed PHGG (44%) than in rats fed the GUAR (30%, $P < 0.02$).

Effects of diets on biliary secretion and fecal excretion of acidic and neutral steroids. As shown in Table 2, the biliary flow was higher in rats fed GUAR and PHGG diets than in controls, and the biliary bile acids secretion was significantly enhanced with rats fed GUAR and PHGG diets (+63% and +36%, respectively, $P < 0.05$), whereas there was no effect on biliary cholesterol secretion. As a consequence, the small intestine bile acids content was markedly enhanced with guar gum diets (89 µmol for GUAR and 70 µmol for PHGG, compared to 50 µmol for controls). In rats fed the guar gum diets, the cecal concentration of bile acids was the same as in control rats (in the range of 7.5–10 µmol/g); nevertheless the cecal pool was significantly enhanced (+100% and +41% with GUAR and PHGG diets, respectively, $P < 0.05$). The bile acids solubility was stongly depressed by acidic pH conditions in the cecum (down to 5–7%), independently of the initial viscosity of guar gum. Bile acids reabsorption in the cecum was 13 µmol/h in controls, and it was significantly enhanced only in rats fed GUAR (up to 21 µmol/h, $P < 0.05$). It

TABLE 1
Effects of Diets on Final Body Weight, Weight of Digestive Organs and Cecal Fermentations[a]

Diets	Final body weight (g)	Cecum weight (g)	Wall weight (g)	Cecal content (g)	Cecal pH	Total SCFA concentration (mmol/L)	SCFA molar ratio %(Ac/Pr/Bu)	Total SCFA pool (µmol/cecum)
Control (0.4% cholesterol)	273 ± 8	2.6 ± 0.1^{a}	0.75 ± 0.03^{a}	1.86 ± 0.09^{a}	7.22 ± 0.05^{b}	79 ± 4^{a}	(66:26:8)	157 ± 9^{a}
8% GUAR (0.4% cholesterol)	253 ± 6	4.5 ± 0.5^{b}	1.15 ± 0.05^{b}	3.35 ± 0.44^{b}	5.95 ± 0.08^{a}	140 ± 9^{b}	(60:30:10)	560 ± 12^{b}
8% PHGG (0.4% cholesterol)	272 ± 6	4.3 ± 0.2^{b}	1.19 ± 0.07^{b}	3.20 ± 0.21^{b}	6.01 ± 0.10^{a}	157 ± 6^{b}	(51:44:5)	545 ± 18^{b}

[a]Each value is the mean ± SEM, $n = 10$. Values within a column that are not followed by a common superscript letter are significantly different ($P < 0.05$). GUAR, natural guar gum; PHGG, partially hydrolyzed guar gum; SCFA, short-chain fatty acid.

TABLE 2
Effects of Diets on Bile and Cecal Bile Acids Concentration, and Their Fecal Excretion in Rats[a]

	Dietary group (0.4% cholesterol)		
	Control	8% GUAR	8% PHGG
Bile			
Flow (mL/h)	1.07 ± 0.11[a]	1.46 ± 0.08[b]	1.38 ± 0.12[b]
Bile acids secretion (µmol/h)	44 ± 5[a]	72 ± 9[b]	60 ± 7[b]
Cholesterol secretion (µmol/h)	0.7 ± 0.1	0.9 ± 0.2	0.7 ± 0.1
Small intestine			
Bile acids content (µmol/intestine)	50 ± 4[a]	89 ± 6[c]	70 ± 5[b]
Cecum			
Bile acids concentration (mmol/L)	9.1 ± 0.5[b]	10.1 ± 0.8[b]	7.5 ± 0.5[a]
Soluble bile acids (%)	32 ± 3[b]	7 ± 2[a]	5 ± 0.5[a]
Bile acid content (µmol/cecum)	17 ± 1[a]	34 ± 3[c]	24 ± 2[b]
Bile acids absorption (µmol/h)	13 ± 1[a]	21 ± 3[b]	18 ± 2[a,b]
Fecal excretion			
Feces weight (g/d)	1.40 ± 0.12[a]	2.13 ± 0.11[b]	2.32 ± 0.32[b]
Bile acids excretion (µmol/d)	30 ± 2[a]	45 ± 2[b]	35 ± 4[a]
Cholesterol excretion (µmol/d)	66 ± 3[a]	81 ± 5[b]	76 ± 4[b]
Coprostanol excretion (µmol/d)	99 ± 4[a]	143 ± 8[b]	114 ± 6[a]
Total steroids excretion (µmol/d)	195 ± 9[a]	269 ± 15[b]	225 ± 11[a]
Digestive steroids balance			
Apparent neutral sterol absorption[b] (µmol/d)	+77 ± 9[c]	+25 ± 2[a]	+55 ± 5[b]
Apparent cholesterol digestibility[c] (%)	32	10	23
Total steroids balance[d] (µmol/d)	+47	−20	+20

[a]Each value is the mean ± SEM, $n = 10$. Values within a column that are not followed by a common superscript letter are significantly different ($P < 0.05$).
[b]Apparent neutral sterol absorption = cholesterol intake (≈245 µmol/d) − fecal excretion of neutral sterols (coprostanol + cholesterol, in µmol/d).
[c]Apparent cholesterol digestibility = apparent neutral sterol absorption (µmol/d)/cholesterol intake (µmol/d) × 100.
[d]Total steroids retention = cholesterol intake − (fecal excretion of neutral sterols + fecal excretion of bile acids). See Table 1 for abbreviations.

must be noted that this reabsorption represented a very similar percentage of biliary secretion (28–30%) in all diet groups.

The daily fecal weight was markedly increased with GUAR or PHGG (+52% or +65%, respectively). Fecal bile acids excretion was significantly increased only in the GUAR group (+50%) compared to controls ($P < 0.01$). Bile acids fecal excretion represented 2.4 to 2.8% of the biliary bile acids secretion in any conditions. Comparisons between biliary secretion and output from the small intestine (large intestine reabsorption + fecal excretion) afford an estimation of small intestine reabsorption: about 30 µmol/h in controls vs. 49 µmol/h in rats fed GUAR and 40 in those fed PHGG.

In parallel, there was a subtantial excretion of sterols (chiefly as coprostanol) amounting to 165 µmol/d in controls. This excretion was significantly enhanced only in rats fed GUAR, up to 224 µmol/d ($P < 0.01$). In taking into account the daily cholesterol intake (about 245 µmol/d), it appears that the apparent cholesterol absorption was markedly depressed by GUAR (−67%, $P < 0.001$) and to a lesser extent by PHGG (−28%, $P < 0.05$); as a result, the apparent cholesterol digestibility was markedly reduced (10% with GUAR, compared to 32% with the control diet). The total steroids retention was +47 µmol/d in controls; it was lower but still positive in rats fed PHGG, whereas a negative value (−20 µmol/d) was found in rats fed GUAR.

Effects of diets on plasma and liver lipid concentration and hepatic enzyme activities. As shown in Table 3, plasma cholesterol concentration was 2.18 mmol/L in control rats; there was no significant cholesterol-lowering effect of PHGG, whereas GUAR was very effective in this respect (−37%). Plasma triglycerides were significantly depressed by both GUAR and PHGG. In rats fed the control diet (0.4% cholesterol), the major part of plasma cholesterol was found in the $d < 1.040$ kg/L fraction (Fig. 1). GUAR was very effective in lowering cholesterol in this fraction (−71%), in contrast to PHGG which was marginally effective. Cholesterol in the $d > 1.040$ kg/L fraction was not significantly modified by any of the diet conditions.

The cholesterol diet elicited a marked accumulation of cholesterol in the liver, essentially as cholesterol esters in controls; only GUAR was effective in lowering liver cholesterol, especially the cholesterol esters fraction (Table 3). In parallel, the triglycerides accumulation was reduced only by the GUAR diet.

The microsomal activity of HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis, was very low in control conditions (≈20 pmol/min/mg protein), owing to re-

TABLE 3
Effects of Diets on Plasma Lipids Concentration and Liver HMG-CoA Reductase or Fatty Acid Synthase[a]

	Plasma		Liver				
Diets	Cholesterol (mmol/L)	Triglycerides (mmol/L)	Total cholesterol (mg/g·liver)	Free cholesterol (mg/g·liver)	Triglycerides (mg/g·liver)	Microsomal HMG-CoA reductase (pmol/min/mg protein)	Fatty acid synthase (nmol/min/g·liver)
Control (0.4% cholesterol)	2.18 ± 0.12[b]	1.41 ± 0.13[b]	7.17 ± 0.95[b]	2.36 ± 0.28[a]	13.65 ± 1.32[b]	22 ± 4[a]	318 ± 10[c]
8% GUAR (0.4% cholesterol)	1.37 ± 0.07[a]	1.22 ± 0.12[a]	2.75 ± 0.16[a]	1.87 ± 0.11[b]	8.62 ± 1.04[a]	124 ± 23[c]	213 ± 12[a]
8% PHGG (0.4% cholesterol)	1.97 ± 0.17[b]	1.07 ± 0.11[a]	6.57 ± 0.58[b]	2.29 ± 0.25[a]	11.45 ± 1.09[b]	52 ± 8[b]	265 ± 13[b]

[a]Each value is the mean ± SEM, $n = 10$. Values within a column that are not followed by a common superscript letter are significantly different ($P < 0.05$). HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA. See Table 1 for other abbreviations.

pression by dietary cholesterol. This activity remained very low with the PHGG diet, whereas there was a striking induction in rats fed the GUAR diet (up to 124 pmol/min/mg protein). The cytoplasmic activity of fatty acid synthase was significantly depressed in rats fed galactomannans (−33% and −16% for GUAR and PHGG, compared to controls).

DISCUSSION

It is now well established that guar gum is a potent fermentable polysaccharide to lower plasma cholesterol (16,17), but one major drawback of guar gum is its very high viscosity, which alters its acceptability (8). Thus, attempts have been made to prepare less viscous forms of guar gum, generally by partial enzymatic hydrolysis. With the present products, viscosity had little influence on several major parameters of cecal fermentations such as cecal hypertrophy, luminal acidification, or total SCFA concentration. Nevertheless, the fact that PHGG elicited fermentations particularly high in propionic acid suggests that MW may affect the rate of substrate fermentation, hence the SCFA molar ratio.

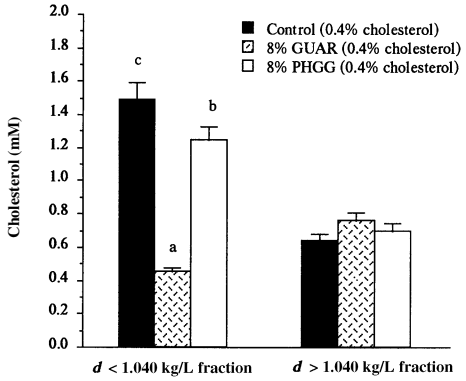

FIG. 1. Changes in concentration of cholesterol (mM) in lipoprotein fractions, in controls rats, and rats adapted to GUAR and PHGG included in 0.4% cholesterol diets. Each value is a mean ± SEM of three pools of rat plasma representing 10 animals per pool. The fractions with a density lower than 1.040 kg/L corresponded chiefly to triglyceride-rich lipoproteins, with a minor contribution of low density lipoprotein. The fractions with a density higher than 1.040 kg/L corresponded essentially to high density lipoprotein. Values not sharing a common letter are significantly different ($P < 0.05$). GUAR, natural guar gum; PHGG, partially hydrolyzed guar gum.

In the present experiment on rats fed 0.4% cholesterol diets, viscous guar gum exerted a strong cholesterol-lowering effect, as previously reported (18,19), whereas this effect is less potent with cholesterol-free diets (20). However, PHGG appears practically devoid of cholesterol-lowering properties in this experiment but displayed a slight but significant triglyceride-lowering effect. In rats fed a cholesterol diet, hypercholesterolemia generally corresponds to a rise in triglyceride-rich lipoprotein cholesterol, HDL cholesterol being practically unchanged (21), and the cholesterol-lowering effect of guar gum affected chiefly the $d < 1.040$ kg/L fraction.

Guar gum is one of the most potent fibers to bind the components of micelles in the small intestine: bile acids, but also phospholipids or cholesterol (6,22). Nevertheless, it is still uncertain whether bile acid availability for dispersion and hydrolysis of lipids is substantially modified. Poksay and Schneeman (23) have reported that, in rats fed guar gum, there is a lowering of bile acids concentration (but the intestinal pool was practically unchanged), whereas Ikegemi *et al.* (24) reported a higher rate of bile acid secretion. The present data show a higher rate of biliary secretion and a larger small intestine pool with GUAR, but it is noteworthy that PHGG also affected these parameters, although less markedly.

The present work suggests that the small intestine reabsorption of bile acids was markedly enhanced in rats fed GUAR (+63%) and to a lesser extent in those fed PHGG (+33%). Yet guar gum has the potential to impair bile acid absorption (5,6,25), but this may be counteracted by opposing factors: enlargement of the small intestine pool and upregulation of ileal transport (26). Nevertheless, even if the ileal reabsorption of bile acids was enhanced in rats fed GUAR, the absorption capacities have probably been overwhelmed since the cecal bile acid pool was dramatically enhanced, as shown for other types of fibers (27). As well as in the small intestine, bile acid reabsorption in the cecum is not sufficient for complete recovery of bile acids escaping ileal reabsorption. Alterations of the physicochemical characteristics of the cecal medium elicited by guar gum fermentation

appear very effective in depressing the solubility of bile acids (from 32% in control conditions down to 5–7% with GUAR or PHGG). Various mechanisms could be invoked: effect of pH itself, elevated availability of Ca, and enlarged bacterial mass, which represents potential binding sites for bile acids (28). However, since the cecal absorption was significantly enhanced with GUAR (and to a lesser extent with PHGG), the consequences of bile acids insolubilization were certainly compensated for by factors such as an accelerated blood flow and an extended surface area of exchange. Whether some other factors could accelerate passive reabsorption itself is still undetermined.

The present experiment indicates that guar gum alters the steroid balance, chiefly by affecting the cholesterol digestibility: with the GUAR diet, compared to control conditions, there was a +59 μmol/d increase in neutral sterol excretion vs. +15 μmol/d for bile acids excretion. Various investigations have established that guar gum is liable to depress cholesterol absorption in the small intestine (19,29), but whether this reflects a direct effect on cholesterol absorption or results from bile acid trapping is still uncertain. As a result, the positive (cholesterol intake–steroid excretion) balance in control rats (+47 μmol/d) was shifted toward negative value (–20 μmol/d) in rats fed GUAR. In rats fed PHGG, this balance was still positive but less than in controls (+20 μmol/d). It appears thus that, in rats adapted to a GUAR diet, there is a concomitant rise in the liver secretion and in the intestinal reabsorption of bile acids, and not merely a diversion of bile acids toward fecal excretion.

PHGG had generally a lesser, but nonnegligible, effect on the various parameters of steroid enterohepatic cycling. Nevertheless, this was not sufficient to shift the steroid balance toward a negative status, required for effective cholesterol-lowering effect.

Cholesterol diets are well known to elicit an accumulation of cholesterol esters and triglycerides in the liver (19,30) and to depress the activity of HMG-CoA reductase drastically (31). It is noteworthy that the cholesterol-lowering effect of GUAR was accompanied by a marked reduction in hepatic cholesterol and the induction of HMG-CoA reductase activity, suggesting that cholesterogenesis could be reactivated by some fibers, as shown by others (32). In the rat, the activity of cholesterol-7α hydroxylase is generally induced by cholesterol feeding, but it may be further induced by steroid sequestrants or gel-forming compounds (3), which is consistent with data on daily bile acids excretion. In rats fed a guar gum diet, this accelerated oxidation of cholesterol toward bile acids apparently leads to an up-regulation of HMG-CoA reductase. It has been proposed that HMG-CoA reductase could be induced in response to a reduced transport of bile acids back to the liver, bile acids being considered as down-regulators of HMG-CoA reductase (33,34). Since bile acid reabsorption is enhanced in rats fed GUAR, this mechanism seems unlikely in the present experiment. Rather, the depletion of liver cholesterol in rats fed GUAR could lead to an up-regulation of the enzyme activity. Inhibition of cholesterol absorption induced by GUAR in the presence of a substantial activity of HMG-CoA reductase appears favorable to normalize plasma cholesterol.

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Lipids of Hamster Cheek Pouch Epithelium

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ABSTRACT: The hamster cheek pouch is a much used but incompletely understood experimental model. In particular, the cheek pouch epithelial lipids, which are important for permeability barrier function as well as other aspects of epithelial biology, have not been completely characterized. In the present study, the complete lipid class composition has been determined by thin-layer chromatography in conjunction with photodensitometry. The major lipid classes were phospholipids, free sterols, and ceramides. Minor amounts of monohexosylceramides, sterol esters, fatty acids, and triglycerides were also present. Significant amounts of covalently bound ω-hydroxyceramide was also detected. Transmission electron micrographs reveal extensive, largely paired, lipid bilayers in the intercellular spaces of the stratum corneum.

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The hamster cheek pouch frequently has been used as a model for studies on oral carcinogenesis (1) and candidiasis (2). From the beginning of 1992 to December 1996, 81 studies have appeared in the MedLine data base that employed the hamster cheek pouch model.

The epithelium in the hamster cheek pouch is a keratinizing epithelium, similar to epidermis or the epithelium from the gingiva and hard palate in the human (3). In these keratinizing epithelia, keratinocytes differentiate to produce a stratum corneum consisting of flat, polygonal cells filled with keratin and surrounded by a lipid matrix (3). The stratum corneum provides a permeability barrier that limits the rate at which materials may penetrate to the underlying tissue. Materials that do traverse the permeability barrier do so by passive diffusion through the intercellular spaces (4–6); therefore, it is the intercellular lipid that determines the quality of the barrier.

In spite of the wide usage of the hamster cheek pouch model and the importance of lipids in epithelial biology and function, the cheek pouch epithelial lipid composition does not appear to have been determined previously. The purpose of the present study was to address this lack of knowledge.

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EXPERIMENTAL PROCEDURES

Epithelial lipid extraction and analysis. Four 7-wk-old golden Syrian hamsters were euthanized by asphyxiation with carbon dioxide, and the cheek pouches were excised. Biopsies were fixed for electron microscopy, and the remaining tissue was immersed for 1 min in 65° water, after which the epidermis could be peeled from the underlying tissue. The tissue was dried by lyophilization.

Epithelial lipids were then extracted with chloroform/methanol 2:1, 1:1, and 1:2 for 2 h each at room temperature (7). The extracts from a given sample were combined, dried under nitrogen, and redissolved in 5 mL of chloroform/methanol (2:1, vol/vol). This solution was washed with 1 mL of 2 M KCl to remove nonlipid contaminants (8), and after thorough mixing and centrifugation, the lower phase was transferred to a clean tube and dried under nitrogen.

Lipid class composition was determined by thin-layer chromatography in conjunction with photodensitometry (7,9). Silica gel G plates (Adsorbosil Plus; Alltech Associates, Deerfield, IL) were washed with chloroform/methanol (2:1, vol/vol) activated, and the adsorbent was scored into 6-mm-wide lanes. Samples and standards were applied 2–3 cm from the bottom edge of the plate with 5-μL glass capillaries, and the chromatograms were developed.

In order to resolve phospholipids, monohexosylceramides and ceramides chromatograms were developed to 11 cm with chloroform/methanol/water (40:10:1, by vol), followed by chloroform/methanol/acetic acid (190:9:1, by vol) to 20 cm, followed by hexane/ethyl ether/acetic acid (70:30:1, by vol) to 20 cm. Individual phospholipids were resolved by development with chloroform/methanol/2-propanol/0.25% KCl/triethylamine, 30:9:25:6:18 to 20 cm (10).

To resolve the less polar lipids including cholesterol, fatty acids, triglycerides, and cholesterol esters chromatograms were developed with hexane to 20 cm followed by toluene to 20 cm followed by hexane/ethyl ether/acetic acid (70:30:1, by vol) to 12 cm.

After development, plates were air dried, sprayed with 50% sulfuric acid, and slowly heated to 220°C on an aluminum slab on a hot plate. After charring was complete, chromatograms were scanned with a photodensitometer. Standards used for identification of lipids as well as for quantitation included triolein, cholesterol, cholesterol oleate, phosphatidylethanol-

amine, and sphingomyelin purchased from Sigma Chemical Company (St. Louis, MO). Also, phospholipids, monohexosylceramides, and ceramides from pig epidermis were used. There are no suitable commercially available substitutes for many of these sphingolipids.

Electron microscopy (Ref. 11). Small samples of pouch tissue (approximately 1 mm^3) from each animal were fixed in 2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate. Tissue was then post-fixed for 1 h in either 0.2% ruthenium tetroxide in 0.1 M cacodylate buffer at pH 6.8 or in 1% osmium tetroxide with 1.5% potassium ferrocyanate in 0.1 M cacodylate buffer at pH 6.8. The specimens were then dehydrated in a series of acetone solutions (30, 50, 70, 95, and 100%) before embedding in Spurr's resin. Ultrathin sections were cut and collected on carbon-stabilized Formvar-coated grids (Tousimis, Rockville, MD). Sections were then stained with uranyl acetate and lead citrate prior to examination in a Zeiss EM 10 electron microscope (Carl Zeiss Inc., Chicago, IL).

Covalently bound lipids (Ref. 12). The extracted epithelium was dried *in vacuo*, and each sample was treated with 5 mL of 1 M sodium hydroxide in methanol containing 10% water by volume at 60° for 1 h. After the reaction mixture had cooled to room temperature, 1 mL of 6 M hydrochloric acid and 5 mL of chloroform were added. After thorough agitation, the mixture was filtered through scintered glass. The chloroform phase was separated and dried under a stream of nitrogen. The residue was dissolved in chloroform/methanol (2:1, vol/vol) and analyzed by thin-layer chromatography as described for the free polar lipids.

RESULTS

Table 1 summarizes the lipid class composition of hamster cheek pouch epithelium. The phospholipids include sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and phosphatidylethanolamine. The monohexosylceramides and ceramides are similar to the analogous lipids from porcine epidermis and include components with mobilities like acylglucosylceramide and acylceramide in the porcine lipid. The nonpolar lipids from hamster epithelium include free sterol, fatty acid, triglyceride, and sterol esters. In addition to the extractable lipids summarized in Table 1, the hamster epithelium also contains covalently bound lipid matching the ω-hydroxyceramide from porcine stratum corneum and accounting for 0.6 ± 0.2 percentage of the dry weight of the tissue. Smaller amounts of free fatty acids and ω-hydroxy acids are also present.

Figure 1 is a transmission electron micrograph showing the intercellular lipid lamellae in the stratum corneum. It is noteworthy that the lamellae often appear to be paired.

DISCUSSION

Although previous investigators have examined phospholipid and sterol metabolism by hamster oral keratinocytes in cell culture (13–15), the present work is the first complete accounting of the lipid class composition from hamster cheek pouch epithelium. In the previous studies (13–15), the incorporation of ^{14}C-acetate into different lipid classes by basal keratinocytes was measured. Unfortunately, these labeling data cannot be converted into a composition for comparison with the present results. In these earlier studies on lipid synthesis, phospholipids, sterols, fatty acids, triglycerides and sterol esters were detected, and all of these lipid classes also were found in the present study. In contrast, glycosylceramides and ceramides were not detected in the basal keratinocyte cultures, whereas these sphingolipids are abundant in cheek pouch epithelium. The apparent absence of the glycosylceramides and ceramides from basal keratinocytes is in accordance with the concept that basal keratinocytes are relatively undifferentiated cells, while the ceramides and glycosylceramides synthesized as keratinocytes differentiate. Lipid class compositions similar to those found for cheek pouch epithelium have been reported for porcine epidermis and keratinized oral epithelium (7); however, the proportions of the different classes found in cheek pouch epithelium, especially the relatively high proportions of the sphingolipids, are closer to those found in epidermis than porcine oral epithelium.

Likewise, the organization of the intercellular lamellae in the stratum corneum is more reminiscent of that seen in porcine (7), human (16), or murine (11) epidermis than of porcine oral stratum corneum. The keratinizing regions of porcine oral ep-

TABLE 1
Lipid Composition[a] of Hamster Cheek Pouch Epithelium

	Animal 1	Animal 2	Animal 3	Animal 4	Mean ± SD
Sphingomyelin	9.3	7.3	11.8	9.2	9.4 ± 1.8
Phosphatidylcholine	23.9	27.5	22.2	24.8	25.6 ± 2.2
Phosphatidylserine	2.9	2.6	1.9	2.0	2.4 ± 0.5
Phosphatidylinositol	3.0	3.6	2.4	2.7	2.9 ± 0.5
Phosphatidylethanolamine	7.4	9.7	11.6	10.8	9.9 ± 1.8
Glycolipids	3.5	3.9	4.6	3.3	3.9 ± 0.6
Ceramides	12.5	12.1	12.2	9.7	11.6 ± 1.3
Sterols	21.4	19.5	19.7	20.3	20.2 ± 0.8
Fatty acids	6.7	6.8	6.7	8.3	7.1 ± 0.8
Triglycerides	4.8	2.8	3.0	4.0	3.7 ± 0.9
Sterol esters	4.6	4.1	3.0	4.0	4.4 ± 0.5

[a]Compositions are presented as weight percentages.

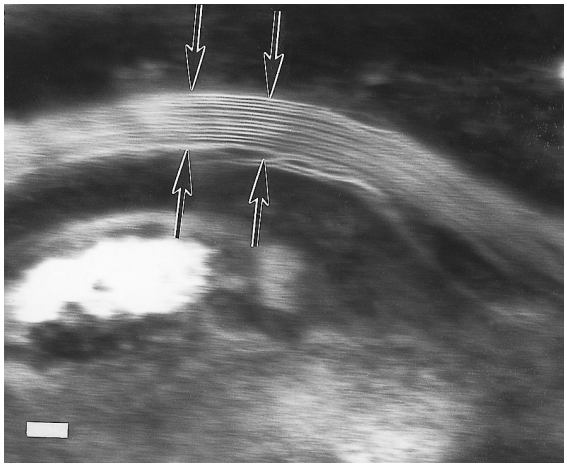

FIG. 1. Transmission electron micrograph of stratum corneum from the hamster cheek pouch. Bar = 50 nm. Note paired bilayers with intervening bands of electron dense material (arrows).

ithelium are adapted for the dissipation of shearing forces associated with mastication, and as such they produce a stratum corneum with many desmosomes. The intercellular spaces between the frequent desmosomes appear to be dilated with a few lamellae at the periphery of the dilation and amorphous material filling most of the interior of the dilation. In contrast, epidermis contains relatively few desmosomes, and the intercellular spaces between the desmosomes in epidermal stratum corneum appear to be more uniform in width and more extensively filled with broad multilamellar sheets.

The transition of membrane structure from the lamellar contents of the membrane coating granules to the broad, multiple lamellae in the stratum corneum in hamster cheek pouch epithelium has been described previously by Hayward (17). The fact that the stratum corneum lamellae appear to be paired can be predicted from Landmann's model for these membrane transitions (18). Based on extensive transmission and freeze fracture electron microscopy, Landmann proposed that the lamellae within the membrane coating granules are actually stacks of flattened lipid vesicles. After extrusion into the intercellular space, the flattened vesicles align and fuse in an edge-to-edge manner to produce paired bilayers.

It has been proposed that in epidermal stratum corneum the covalently bound lipid layer on the outer surface of the corneocyte envelope may serve as a template upon which the flattened vesicles derived from the membrane coating granules can align and fuse to form the extensive broad lamellae that fill most of the intercellular space (19). In keratinized oral epithelia from the porcine hard palate or gingiva, it has been demonstrated that there is no covalently bound lipid, and accordingly, in these tissues there are less extensive and poorly organized intercellular lamellae. The finding of significant levels of covalently bound hydroxyceramide in the present study and the similarity between the intercellular spaces of the cheek pouch stratum corneum and epidermis are in accordance with this proposed role of the covalently bound lipid layer.

It is noteworthy that the intercellular lamellae in hamster cheek pouch stratum corneum can be visualized as well after fixation with osmium tetroxide as with ruthenium tetroxide. This is not the case with epidermal stratum corneum from several different species. With porcine epidermis, for example, the intercellular spaces appear electron lucent without a trace of lamellar structure, following conventional fixation with osmium tetroxide (16). The chemically more reactive ruthenium tetroxide appears to be required to reveal the lamellar organization of the intercellular lipid. This has been shown to be related to the relatively unreactive lipids present in epidermal stratum corneum (16). The present results, as well as one previous study, imply that the lipids in hamster cheek pouch stratum corneum contain more reactive groups than the lipids in epidermal stratum corneum. Future studies will address this point.

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Dietary Sandalwood Seed Oil Modifies Fatty Acid Composition of Mouse Adipose Tissue, Brain, and Liver

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ABSTRACT: Sandalwood (*Santalum spicatum*) seed oil, which occurs to about 50% of the weight of the seed kernels, contains 30–35% of total fatty acids (FA) as ximenynic acid (XMYA). This study was designed to obtain basic information on changes in tissue FA composition and on the metabolic fate of XMYA in mice fed a sandalwood seed oil (SWSO)-enriched diet. Female mice were randomly divided into three groups, each receiving different semisynthetic diets containing 5.2% (w/w) fat (standard laboratory diet), 15% canola oil, or 15% SWSO for 8 wk. The effects of SWSO as a dietary fat on the FA composition of adipose tissue, brain, and liver lipids were determined by analyses of FA methyl ester derivatives of extracted total lipid. The FA compositions of the liver and adipose tissue were markedly altered by the dietary fats, and mice fed on a SWSO-enriched diet were found to contain XMYA but only in low concentration (0.3–3%) in these tissues; XMYA was not detected in brain. Oleic acid was suggested to be a principal XMYA biotransformation product. The results were interpreted to suggest that the metabolism of XMYA may involve both biohydrogenation and oxidation reactions.

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Sandalwood (*Santalum spicatum*) is a small tree or shrub native to the Western Australian arid region. It bears significant numbers of a large fruit, which is a drupe containing a hard-shelled seed which is rich in a drying fixed oil (50–60%, w/w) (1). The kernel of the seed has been part of the traditional Western Australian Aboriginal diet (2) and also has been a minor source of food to people living and working in the bush (3). The food value of the kernel is similar to other commercially available nuts (3).

A distinctive feature of sandalwood seed oil (SWSO) is the presence of conjugated acetylenic fatty acids (FA) with ximenynic acid (XMYA) [30–35% of total FA; *trans*-11-octadecen-9-ynoic acid, 18:2(9a, 11*t*), where "a" indicates the triple-bond position; "*t*" indicates the *trans* configuration of double bond] being the most abundant (1). Stearolic acid [1% of total FA; 9-octadecynoic acid, 18:1(9a)] also occurs naturally (4). The chemistry of conjugated enyne FA is of current interest because of their potential bioactivity as lipoenzyme inhibitors or their involvement in FA metabolism (5–8).

Although kernels of the sandalwood seeds have been eaten in the past without apparent harm, concern has been expressed about the possible long-term toxicity of the acetylenic FA (5–9). The metabolism of XMYA has been previously studied by feeding rats with a diet enriched in quandong (*Santalum acuminatum*) seed oil, which contains XMYA at up to 40–45% of total FA (1,9). Those results indicated that XMYA was incorporated into different tissues only to a minor extent (0.5–5.4%), and it increased hepatic cytochrome P-450 activity (9). Little specific information is currently available about the metabolic fate of XMYA in animals or humans. This study was designed to obtain basic information on quantitative changes in tissue FA composition induced by XMYA and to provide further information on the metabolic utilization of XMYA in mice fed SWSO.

MATERIALS AND METHODS

Diets were formulated and compounded by Glen Forrest Stockfeeders (Perth, Western Australia, Australia). The diets were prepared as 11-mm diameter pellets based upon a sodium caseinate semisynthetic formula with added vitamins and minerals compounded according to the AIN 1993 base formula (10). Canola oil (CO) was purchased from a local supermarket and SWSO was supplied from this study program, following Soxhlet extraction of the mature seed kernels using hexane solvent with complete removal of solvent at reduced pressure using rotary evaporation at 60°C. The diet pellets were stored in closed containers at room temperature. The FA compositions of the diet pellets were determined by gas chromatographic/mass spectral (GC/MS) analysis of the FA methyl esters (FAME).

Animals and diets. Six-week-old female mice, strain ARC, weighing 20 to 30 g, were obtained from the Animal Resources Centre (W.A., Australia) and were given free access to the standard laboratory diet (SLD) for 4 wk. The mice were then randomly divided into three groups, each of 15 mice, and were then ear-punched with unique codes. The mice were

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Abbreviations: AA, arachidonic acid; CO, canola oil; DHA, docosahexaenoic acid; DMOX, dimethyloxazoline; FA, fatty acids; FAME, fatty acid methyl esters; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; SLD, standard laboratory diet; SWSO, sandalwood seed oil; XMYA, ximenynic acid.

housed in standard lab cages, in a humidity (50 ± 5%), temperature-regulated (21 ± 1°C), light-controlled room which was illuminated daily for a 12-h period. The mice were given free access to feed and water at all times. Consumption of diet pellets was determined on a weekly basis, for a total test feeding period of 8 wk.

One group of mice (control) were fed SLD, another group (high-fat test control) was fed the CO-enriched diet, and the third group was supplied the experimental SWSO-enriched diet. At the end of the 8-wk feeding period, the mice were fasted overnight and killed by cervical dislocation. Adipose tissue, brain and liver were quickly removed, washed with physiological saline, and stored at −20°C in closed, labeled tubes for further analyses. The experimental protocol was reviewed and approved by the Animal Experimentation Ethics Committee of Curtin University (Perth, Australia) (approval number: N44/94).

Lipid analysis. Total lipid was extracted from homogenized organ tissue by the method of Bligh and Dyer (11). The dietary oils and the total lipids of various tissues were converted to FAME by the method of Christie (12). The FAME derivatives were characterized and quantified using gas chromatography (GC) on a Hewlett-Packard (Palo Alto, CA) 5890 Series 11 GC system with HP 5971 mass selective detector. The separation was carried out on a DB 23 capillary column (30 m × 0.25 mm i.d., 0.15 μm; J&W Scientific, Folsom, CA) as reported previously (13).

Statistical analysis. Statistical analysis of results was carried out using one-way analysis of variance and the Scheffe's Test (SPSS; SPSS Inc., Chicago, IL) where appropriate.

RESULTS AND DISCUSSION

The summary composition of the three diets is shown in Table 1. FA analysis of the three diets determined as relative percentage FA content is shown in Table 2. The consumption of the three diets was determined on a weekly basis and found to be SLD: 3.85; CO: 3.00; and SWSO: 2.24 g per mouse per day. Inspection of Table 1 data and the available energy for each diet suggest that the mice consumed 0.056, 0.054, and 0.040 MJ, respectively, available energy per day through the various diets. To a certain extent, the reduced food consumption was balanced by the higher energy content of the diet. At the conclusion of this study, the mice had all shown an increase in body weight which varied according to the diet: SLD: 15.78%; CO: 19.62%; and SWSO: 11.45% body weight increase. Thus, a general effect of feeding the SWSO diet was to decrease food consumption, providing a decrease in available energy intake, and this resulted in the lowest body weight increase over the test period of all three diets. There were no mouse deaths during the feeding experiment.

The mice had initial weights of about 26.5 g and gained weight whether they were fed on SLD or CO or SWSO diets. The decreased growth rates of the SWSO group may be due to one or more of a number of factors such as poor utilization of the diet, a growth-retarding action due to an unbalanced ratio of saturated FA (SFA) to monounsaturated FA (MUFA) in dietary fat, or perhaps a specific action of XMYA as an antifeedant FA. A previous feeding study has shown the optimal ratio of SFA to MUFA in diet fat to be 1:0.7 to 1:0.2, and when the ratio was decreased to 1:1.8, a lower growth rate was observed (14). In this study the ratios of SFA to MUFA were determined to be SLD: 1:0.72; CO: 1:10.86; and SWSO: 1:9.72. Clearly, both of the high-fat CO and SWSO diets are far less than the optimal ratio and may thus be expected to produce a lower growth rate as a consequence. However, it was observed that the CO diet produced the greatest increase in body weight, while the SWSO diet produced the lowest growth rate. No specific essential FA-deficiency symptoms were observed in the SWSO-fed animals, suggesting that XMYA had not exerted an antimetabolic effect or inhibited the uptake of essential FA.

The FA compositions of selected mice tissues were determined.

Adipose tissue. Adipose tissue is considered as a dynamic focal point of lipid metabolism (15). Several reports have provided convincing evidence that the FA profile of adipose tis-

TABLE 1
Percentage Composition of Standard and Test Diet Pellets[a]

Component	SLD	CO	SWSO
Protein	19.00	18.35	18.35
Fat	5.20	15.00	15.00
Cellulose	5.00	5.00	5.00
Starch	55.15	45.15	45.15
Sucrose	10.00	10.00	10.00
Crude fiber	5.00	5.00	5.00
Digestible energy (MJ kg^{-1})	14.47	17.85	17.85

[a]All diets were identical in choline chloride (0.1%), DL-methionine (0.15%), mineral mix (AIN 89, 3.5%), and vitamin mix (AIN 89, 1.00%) contents; these were compounded by Glen Forrest Stockfeeders (Perth, Western Australia, Australia) following the AIN 89 guidelines. SLD, standard laboratory diet; CO, corn oil; SWSO, sandalwood seed oil.

TABLE 2
Relative Percentage Composition of Fatty Acids in Standard and Test Diets[a] (g/100 g total FA)

Fatty acid	SLD	CO	SWSO
14:0	1.09	n.d.	n.d.
16:0	33.73	4.16	3.53
16:1n-7	0.69	trace[b]	0.15
18:0	8.09	2.38	1.92
18:1n-9	30.24	71.05	52.83
18:2n-6	24.88	18.89	1.86
18:3n-3	1.29	3.51	3.52
18:1(9a)	n.d.	n.d.	1.51
XMYA	n.d.	n.d.	34.68
Subgroups			
Total SFA	42.91	6.54	5.45
Total MUFA	30.92	71.05	52.98
Total polyunsaturated FA	26.17	22.40	5.34
Total acetylenic FA	n.d.	n.d.	36.19

[a]n.d. = not detected; XMYA, ximenynic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acids; FA, fatty acid.
[b]Trace (<0.01%). See Table 1 for other abbreviations.

sue usually reflects dietary FA composition (16,17). Thus, FA analysis of adipose tissue may provide important information on the uptake and utilization of SWSO, specifically with regard to its FA composition. The effects of SLD, CO, and SWSO dietary fat on adipose tissue FA composition in mice are presented and compared in Table 3.

We have observed that the adipose tissue relative FA composition did not necessarily reflect the dietary FA composition and, indeed, quite profound changes were observed. The relationship between tissue and dietary individual FA profiles has been quantified by calculating the ratio: tissue FA/diet FA, × 100 of specific FA. A strongly positive value in excess of 100 may indicate either enrichment with respect to that FA, evidence of synthesis of one FA from another dietary FA, or differences in intestinal uptake of specific FA. Conversely, a low value, less than 100, may indicate metabolism of the dietary FA and hence its low incorporation into adipose tissue or reduced intestinal uptake of that FA.

It was found that the major SFA of adipose tissue was 16:0, and 18:1n-9 was the predominant MUFA in adipose tissue of all groups. Earlier studies have pointed out that 18:1n-9 is certainly the most widespread FA, representing in many fats more than 30% of the total FA (18).

Mouse consumption of the SLD resulted in decreased SFA components. In contrast, there were marked increases in MUFA such as 16:1n-7 and 18:1n-9, with the appearance of vaccenic acid (18:1n-7) as a minor "new" entity in the adipose tissue. Vaccenic acid is a common FA in laboratory animals, being formed by microorganisms present in the intestine of mice and it may also arise during coprophagy (19).

Mice fed the CO-diet showed a marked increase in 16:0, with slight relative decreases in 18:1n-9, 18:2n-6 and 18:3n-3 compared with their dietary fat supplement composition. Mice fed the experimental SWSO-diet showed a marked relative increase in 16:0, together with a significant relative increase in 18:1n-9 in comparison with the dietary composition. A relative increase was observed in 16:1n-7 level and a marked decrease in 18:3n-3 relative content.

In general, 16:1n-7 occurred only to a low relative content in the dietary fats e.g., 0.7% in SLD, and only traces in CO and SWSO diets. Thus, the large relative increase of this acid in the body fat in all feeding groups was of considerable interest, especially because adipose tissue is the important site for FA biosynthesis. For example, SFA such as 18:0 can be a precursor for conversion into the corresponding monounsaturated form, 18:1n-9 by microsomal Δ9-desaturase enzyme (20,21). Higon *et al.* (21) have demonstrated that such enzymatic reactions may be expressed by the calculated 16:1/16:0 and 18:1/18:0 ratios, and a rise in the numerical value of these ratios would indicate a higher lipogenic activity.

This study demonstrated that the SWSO-fed mice had significantly increased ($P < 0.05$) 16:1/16:0 and 18:1/18:0 ratios, suggesting that XMYA, the major differentiating factor between CO and SWSO diets, may stimulate Δ9-desaturase enzyme activity, an observation which will require further investigation. This finding may be contrasted with the observation that a CO-diet containing *ca*. 71% 18:1n-9 relative FA content did not increase the adipose tissue level of 18:1n-9 but rather maintained it at approximately the same level. In contrast, an earlier study has reported that feeding rats a diet containing high 18:1n-9 resulted in an increased percentage of 18:1n-9 in adipose tissue with a decreased percentage of 16:0 (22).

XMYA constituted 34% of the FA in the SWSO diet, but only about 3% of total FA could be detected in adipose tissue lipids. This is approximately 10% of its content in SWSO diet. This relatively constant relationship, detected in all the adipose tissue samples of rats fed the SWSO diet, may reflect rapid metabolism of XMYA after ingestion, or that XMYA was simply not absorbed.

TABLE 3
Relative Percentage Composition of Fatty Acids in Mouse Adipose Tissue Lipid[a] (g/100 g total FA)

Fatty acid	SLD	T/D	CO	T/D	SWSO	T/D
14:0	0.81 ± 0.07[b]	74.3	trace	—	0.61 ± 0.05[a]	—
16:0	24.66 ± 2.00[b]	73.1	14.41 ± 2.28[a]	346.4	14.80 ± 1.05[a]	419.3
16:1n-7	4.85 ± 1.70[b]	702.9	2.36 ± 0.77[a]	23600	4.75 ± 0.81[b]	3166.7
18:0	1.88 ± 0.52[b]	23.23	1.34 ± 0.33[b]	56.30	0.54 ± 0.20[a]	28.10
18:1n-9	53.28 ± 2.79[a]	176.2	65.75 ± 1.29[b]	92.50	70.71 ± 1.03[c]	133.8
18:1n-7	2.38 ± 0.39[b]	—	1.90 ± 0.24[a,b]	—	1.54 ± 0.29[a]	—
18:2	12.14 ± 1.2[b]	48.8	12.65 ± 1.29[b]	67.00	2.49 ± 0.58[a]	133.8
18:3	trace	0.80	1.59 ± 0.02[b]	45.3	0.45 ± 0.11[a]	12.80
18:1(9a)	n.d.	—	n.d.	—	0.82 ± 0.19	54.30
XMYA	n.d.	—	n.d.	—	3.29 ± 0.86	9.50
Subgroups						
16:1/16:0	0.19 ± 0.07[a]		0.12 ± 0.04[a]		0.32 ± 0.07[b]	
18:1/18:0	29.78 ± 7.29[a]		51.35 ± 11.89[b]		118.2 ± 14.29[c]	

[a]Values represent means ± SD of five determinations. Same superscript letters indicate samples which are not significantly different ($P < 0.05$) within a row using the one-way analysis of variance. T/D = tissue FA/dietary FA × 100; n.d. = not detected. See Table 1 for other abbreviations.

Consideration also should be given to the stereochemistry of the XMYA C_{11}-C_{12} double bond which is of a *trans* configuration. In animal studies it has been found that the deposition of *trans* FA in adipose tissue is dependent on its concentration in the dietary fat intake (23). For example, feeding rabbits for 3 wk a diet providing 40% of energy from fat of which 38% was derived from elaidic acid (*trans* 18:1n-9*t*) resulted in the rabbit adipose tissue containing 22% of this *trans* FA (22). In an earlier study (24), it was noted that the amount of 18:0 was higher in adipose tissue in methyl-elaidate-1-^{14}C-fed fat-deficient guinea pigs than those of coconut-oil-fed animals. The pathway leading to the high level of 18:0 was considered to occur by direct biohydrogenation of elaidic acid. XMYA is an example of an unusual *trans* FA which showed insignificant incorporation.

In conclusion, the marked increase in 18:1n-9 content in SWSO-fed mice adipose tissue could be the result of at least two processes: in the first XMYA stimulates the activity of Δ9-desaturase enzyme to convert 18:0 into 18:1n-9, and in the second XMYA could be the substrate for 18:1n-9 biosynthesis *via* a biohydrogenation process. Therefore, the observed high 18:1/18:0 ratio of SWSO-fed mice (118.2 ± 14.29) may have resulted from a mechanism [1], and the higher relative content of 18:1n-9 also would seem to support the role of XMYA as a precursor in another mechanism [2].

$$18{:}0 \xrightarrow[\text{ximenynic acid}]{\text{9–desaturase}} 18{:}1\text{n–}9 \qquad [1]$$

$$18{:}2(9\text{a}, 11t) \xrightarrow{\text{biohydrogenase}} 18{:}1(9c, \text{or } 11t) \qquad [2]$$

Previous workers have indicated that the biohydrogenation of double bonds in nature is only found in ruminant animals and as a reaction in some bacteria, and that "conjugated linoleic acid" (CLA, *cis*-9, *trans*-11-octadecadienoic acid) may be formed by isomerization during hydrogenation of linoleic to stearic acid (25). It may be that biotransformation of XMYA involves an initial step of biohydrogenation of its C9,10-yne bond to form a CLA which is further biohydrogenated to oleic or vaccenic acids.

Stearolic acid, present in SWSO only in low concentration (1%), also was detected in low concentrations (0.8%) in adipose tissue. No evidence has been reported in the literature for biohydrogenation of XMYA to stearolic acid.

Dietary FA content may have a bearing on adipose tissue FA content, but the depositional effect reflects more the actual nature of the FA rather than its general abundance. In this study the adipose tissue FA composition was not a consistent indicator of diet FA content although the content of certain FA in adipose tissue lipid was significantly affected by the dietary intake. It may also be a point of conjecture, but not proven by this study, that an apparent requirement of the animal is to maintain at least a minimal level of 18:1n-9 content, especially in adipose tissue.

Brain. There was no effect on brain FA composition of feeding the different diets, and there was a remarkable degree of similarity between the samples. This may suggest that mouse physiology controls brain lipid FA composition to an exact degree, possibly almost as a protective mechanism for this center of cerebral activity. XMYA was found to be absent from the brain lipid fraction which was in accordance with a recent study (9). The authors suggested that if rats were fed during the critical period of brain development, it could result in the incorporation of XMYA into brain lipids. Other earlier studies have also indicated that the acetylenic FA, crepenynic and stearolic acids, were not incorporated into brain lipids (26).

Liver. The effects of different diets on mouse liver FA composition are shown in Table 4. The liver FA composition of mice fed the SLD almost reflected dietary 16:0, 18:0, and 18:1n-9 levels. The major observed result of the SLD was a relative 50% decrease in the 18:2n-6 level in the liver, accompanied by the appearance of longer chain FA such as arachidonic acid (AA) and docosahexaenoic acid (DHA). Mice fed the CO diet had increased levels of 18:0 and decreases in 18:1n-9 relative content when compared to those on SLD and SWSO diet. The increase in 18:0 did not reflect the original low level in the CO diet. The SWSO-fed mice demonstrated the highest levels of 18:1n-9 in the liver together with a marked increase in the proportion of 18:1(11*t*). The possible reasons for such a relative increase in 18:1n-9 were discussed previously for adipose tissue.

The liver is the important organ for FA oxidation and biosynthesis (25,27), where SFA such as 16:0 and 18:0 can be converted to the corresponding MUFA 16:1n-7 and 18:1n-9 by microsomal Δ9-desaturase enzyme activity (27).

All mice in this study showed evidence of FA elongation and desaturation processes in the liver. Compared with the control SLD, mice fed the CO diet had significantly higher relative levels of AA, suggesting that more active processes of elongation and desaturation of 18:2n-6 may have occurred.

TABLE 4
Relative Percentage Composition of Fatty Acids in Mouse Liver Lipid[a] (g/100 g total FA)

Fatty acid	SLD	CO	SWSO
16:0	31.51 ± 1.33[b]	27.22 ± 2.77[a,b]	24.61 ± 2.00[a]
16:1n-7	2.04 ± 0.71[a]	1.26 ± 0.77[a]	2.14 ± 0.89[a]
18:0	8.95 ± 0.83[a]	13.11 ± 2.02[b]	6.28 ± 2.60[a]
18:1n-9	34.64 ± 2.87[b]	26.61 ± 2.67[a]	54.35 ± 2.12[c]
18:1n-7	1.76 ± 0.44[a]	1.73 ± 0.26[a]	2.27 ± 0.60[a]
18:2n-6	12.42 ± 2.13[b]	10.69 ± 2.58[b]	3.89 ± 0.52[a]
18:3n-3	0.19 ± 0.16[a]	0.76 ± 0.15[b]	0.24 ± 0.20[a]
AA	6.00 ± 1.45[b]	12.43 ± 1.83[c]	1.84 ± 0.57[a]
DHA	2.49 ± 0.72[a]	6.19 ± 0.87[b]	3.897 ± 1.64[a]
XMYA	n.d.	n.d.	0.24 ± 0.04
Unknown	n.d.	n.d.	0.27 ± 0.13
Subgroups			
DHA/AA	0.42[a]	0.46[a]	2.10[b]

[a]Values respresent means ± SD of five determinations. Same superscript letters indicate samples which are not significantly different ($P < 0.05$) within a row using the one-way analysis of variance; n.d. = not detected. AA, arachidonic acid; DHA, docosahexaenoic acid. See Tables 1 and 2 for other abbreviations.

In contrast, the lower levels of AA of SWSO-fed mice may indicate that XMYA competitively inhibit the conversion of 18:2n-6 to AA. This finding is supported by an earlier study in which rats fed a control diet containing 0.5% of 5,8,11,14-eicosatetraynoic acid showed not only that this acid affected the Δ9-desaturase system but also inhibited the elongation of unsaturated FA (28). However, compared with the control SLD group, mice fed the SWSO diet had higher levels of DHA, which may suggest that while XMYA inhibits the conversion of AA, it promotes the formation of DHA. DHA has the potential to antagonize AA metabolism and hence influence the synthesis of AA-derived inflammatory mediators (29). Therefore, such an effect may have future therapeutic significance to reduce inflammatory responses while promoting the formation of beneficial FA such as DHA.

In conclusion, this study has demonstrated that female mice fed a SWSO-enriched diet showed marked relative increases of 16:0 and 18:1n-9 content in adipose and liver tissues in comparison to SLD and CO-enriched diets. Little change was observed in brain tissue FA relative content. Little XMYA was observed to be incorporated into any of the tissues analyzed, reflecting either a high metabolism of XMYA or simply lack of significant absorption from the intestine. Interpretation of the effects of SWSO on tissue FA relative concentrations suggested the possibility of XMYA undergoing metabolic biohydrogenation (through suitable CLA intermediates) or by stimulation of Δ9-desaturase enzymes. A significant finding was the marked relative increase in liver tissue DHA content with a decrease in the relative content of AA.

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The Unusual Occurrence of 14-Methylhexadecanoic Acid in Pinaceae Seed Oils Among Plants

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ABSTRACT: 14-Methylhexadecanoic (14-MHD) acid has been identified in a sample of pine seed oil (*Pinus contorta*) by gas–liquid chromatography–mass spectrometry of its picolinyl ester derivative. Its identification (through its equivalent chain length) and its distribution in four conifer families have been checked. It occurred only in Pinaceae, where it was found in 72 species belonging to the genera *Pinus*, *Abies*, *Cedrus*, *Tsuga*, *Pseudotsuga*, *Larix*, and *Picea*, in the range 0.02–1.15%. 14-MHD acid could not be detected in the lipids of Taxaceae (*Taxus baccata*), Cupressaceae (*Juniperus communis*), or Taxodiaceae (*Sciadopytis verticillata*), even after a 10-fold concentration of the saturated acid fraction isolated by argentation thin-layer chromatography. It is concluded that Pinaceae, along with *Ginkgo biloba* seed lipids, are major exceptions in the plant kingdom with regard to 14-MHD acid, which otherwise occurs almost exclusively in lipids of animals and microorganisms. The biosynthesis and metabolic role of 14-MHD acid, which otherwise also occur in wood and leaf lipids, remain unknown.
Lipids 32, 971–973 (1997).

It is generally assumed that methyl-branched saturated fatty acids are characteristic components of animal and microbial lipids, and that they generally do not occur in plant lipids. However, 14-methylhexadecanoic (14-MHD, or *anteiso*-17:0) acid was recently formally identified in *Ginkgo biloba* (maidenhair tree, a Gymnosperm) seed lipids by mass spectrometry (MS) of its picolinyl ester derivative (1). In an earlier study of this material and of other conifer seed lipids, Takagi and Itabashi (2) tentatively identified a 16:2n-6 acid that eluted during gas–liquid chromatography (GLC) on a Silar 5CP capillary column (commercial source not given in Ref. 2) between the 16:1 isomers and 17:0 acid, and which was present in higher amounts in *G. biloba* than in other Gymnosperm species. Because this is the place where 14-MHD acid is expected to elute, one may retrospectively surmise that the 16:2n-6 acid might have been confused with 14-MHD acid. However, this is not certain, because Jamieson and Reid (3) observed and tentatively identified both the 16:2n-6 and 14-MHD acids in the needle lipids of a large collection of conifer species. In addition to these acids, these authors also reported on the presence of *trans*-3-16:1 and 16:3n-3 acids that elute in the same region. 14-MHD acid was also formally identified by its mass spectrum in the sapwood of several North American pines (4).

In two systematic studies of conifer seed lipids, Wolff and Bayard (5) and Wolff *et al.* (6) did not mention any component eluting between the two close-eluting 16:1 isomers and 18:0 acid on a CP-Sil 88 capillary column (Chrompack, Middelburg, The Netherlands). Only recently was it recognized (7,8), through the use of a DB-Wax capillary column (J&W Scientific, Folsom, CA), that there existed an acid that was distinct from the 16:1 isomers and that eluted between them and 17:0 acid. This unknown component coeluted with the second-eluting 16:1 isomer on the CP-Sil 88 capillary column, and it was erroneously reported as a 16:1 acid in earlier publications (5,6). Later, it was observed that the unknown acid migrated along with the saturated acid fraction after silver-ion thin-layer chromatography (Ag-TLC) (7). In addition, it coeluted on both the CP-Sil 88 and the DB-Wax capillary columns with an authentic commercial standard of 14-MHD acid methyl ester (7), and it was then tentatively identified as 14-MHD acid. Although it is absent from Angiosperm lipids, we demonstrate here, by GLC–MS of its picolinyl ester derivative, that the unknown component is actually 14-MHD acid, and that it occurs in the seed oil of numerous conifer species, but exclusively of the Pinaceae family.

EXPERIMENTAL PROCEDURES

Samples. Fatty acid methyl esters (FAME), prepared in duplicate from the seed oil of several conifer species, were available from previous studies by one of us (5–8). The origin of the seeds, the extraction of oils, and the preparation of FAME have been described in detail elsewhere (5–8).

Analytical GLC. FAME were analyzed in a Carlo Erba 4130 chromatograph (Carlo Erba, Milano, Italy) equipped with a DB Wax column (30 m × 0.32 mm i.d., 0.5 μm film; J&W Scientific). The oven temperature was 190°C, and the inlet pressure of the carrier gas (helium) was 140 kPa. The injector (split mode) and the flame-ionization detector were maintained at 250°C. Quantitative data were calculated by an

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Abbreviations: Ag-TLC, argentation thin-layer chromatography; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; 14-MHD acid, 14-methylhexadecanoic acid; MS, mass spectrometry.

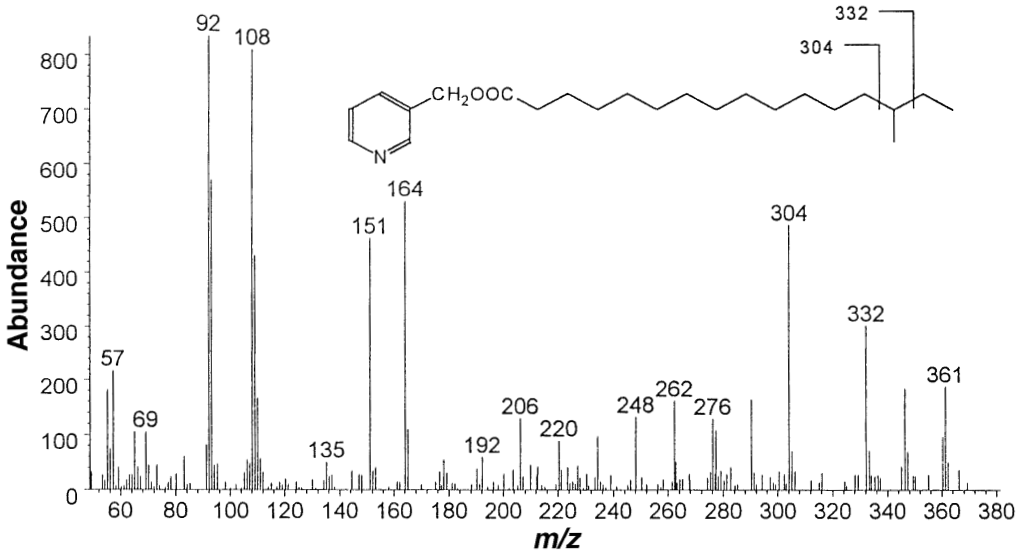

FIG. 1. Mass spectrum of picolinyl 14-methylhexadecanoate.

SP 4290 integrator (Spectra Physics, San Jose, CA). Identification of fatty acids was made as described previously (5,6).

GLC–MS. Methyl esters were hydrolyzed to the free fatty acids (9) before conversion to the picolinyl ester derivatives as described by Balazy and Nies (10). The derivatives were submitted to GLC–MS, with a Hewlett-Packard 5890 Series II plus gas chromatograph (Palo Alto, CA) attached to an HP model 5989 MS apparatus. The latter was used in the electron impact mode at 70 eV with a source temperature of 250˚C. The chromatograph was fitted with on-column injection, and equipped with a capillary column of fused silica coated with BPX-70 (50 m × 0.22 mm i.d., 0.25 µm film; SGE (UK) Ltd., Milton Keynes, United Kingdom). After holding the temperature at 80˚C for 3 min, the column was temperature-programmed at 20˚C/min to 160˚C, then at 2˚C/min to 260˚C, where it was held for 5 min. Helium was the carrier gas.

RESULTS AND DISCUSSION

Identification of 14-MHD. The methyl and picolinyl ester derivatives of the fatty acids were examined by GLC–MS, using a polar BPX-70 column for the separation. The spectrum of the picolinyl ester derivative is shown in Figure 1. The molecular ion is at *m/z* = 361, confirming the molecular weight, but the key diagnostic feature is a gap of 28 amu between *m/z* = 304 and 332. This represents cleavage on either side of the methyl branch point and is typical of cleavage expected of an *anteiso* derivative (11). The mass spectrum of the methyl ester was consistent with this interpretation (9).

Distribution of 14-MHD. As shown in Table 1, 14-MHD acid (identified on chromatograms by its equivalent chain length, 16.68) was present in all Pinaceae species analyzed. It was in the range 0.02–0.23% in *Pinus* species, 0.52–0.57 in *Tsuga* species, 1.15% in *Pseudotsuga menzieseii*, 0.17–0.26% in *Picea* species, 0.42–0.98% in *Cedrus* species, 0.57–0.87% in *Abies* species, and 0.36–0.42% in *Larix* species. Thus it seems that different species inside a given genus show some similarities with regard to their 14-MHD acid level. On the other hand, for species from other conifer families (Taxaceae, Cupressaceae, and Taxodiaceae; results not shown), 14-MHD acid was either absent or at the limit of detection. This was confirmed by GLC analysis after a 10-fold concentration of the saturated acid fractions prepared by Ag-TLC from the seeds of *Taxus baccata*, *Juniperus communis* and *Sciadopytis verticillata*, each of these species being representative of one of the preceding families (results not shown). Not even a trace of 14-MHD acid could be detected in these species, although 17:0 acid was clearly present (results not shown). It should be emphasized that 14-MHD acid was also very low in the needle lipids from these families (0.1–0.4%) as compared to Pinaceae (0.2–4.8%) (3). The reason why 14-MHD acid is present in the leaves and not in the seeds is unexplained.

This study has clearly demonstrated that 14-MHD acid is not limited to animals or microorganisms. It is a common constituent of the needle (3), wood (4), and seed (this study) lipids of Pinaceae. Though the amount of 14-MHD acid was low (most often less than 1%), it was present in the seed lipids from all of the 72 species studied here. However, no other branched-chain component was detected, to the contrary of some conifer leaf lipids where 12-methyltetradecanoic acid has been tentatively identified in trace amounts (3).

The peculiar distribution of 14-MHD acid in conifer seed lipids indicates that the presence of this constituent cannot be attributable to chance. This distribution precludes any possibility of contamination by microorganisms of some seed lots. However, to exclude this possibility definitively, we have purified the triacylglycerols from the crude seed oil of one species (*P. contorta*), and we have prepared FAME therefrom, because it is known that branched acids in microorganisms are esterified to polar lipids. We found that 14-MHD acid was present in triglyceride FAME at the same concentration as that in the original crude oil (result not shown).

TABLE 1
14-Methylhexadecanoic Acid (14-MHD) Content of Pinaceae Seed Lipids (wt% of total fatty acids, mean)

Species	14-MHD	Species	14-MHD	Species	14-MHD
Pinus banksiana	0.17	*P. laricio*	0.15	*P. abies*	0.20
P. contorta	0.23	*P. koekelare*	0.11	*P. sitchensis*	0.17
P. palustris	0.16	*P. nigra*	0.13	*P. glauca*	0.24
P. elliotii	0.17	*P. massoniana*	0.12	*Cedrus atlantica*	0.98
P. caribaea	0.18	*P. mughus*	0.07	*C. atlantica glauca*	0.76
P. echinata	0.16	*P. cembra*	0.09	*C. libani*	0.51
P. occidentalis	0.12	*P. peuce*	0.08	*C. deodara*	0.42
P. attenuata	0.11	*P. monticola*	0.12	*C. brevifolia*	0.70
P. muricata	0.16	*P. parviflora*	0.07	*Abies lasiocarpa*	0.70
P. radiata	0.11	*P. strobus*	0.06	*A. pinsapo*	0.65
P. taeda	0.15	*P. sibirica*	0.07	*A. balsamea*	0.57
P. pinaster	0.20	*P. koraiensis*	0.06	*A. cephalonica*	0.76
P. ponderosa	0.16	*P. griffithii*	0.15	*A. fraserii*	0.67
P. michoacana	0.15	*P. canariensis*	0.05	*A. equi-trojanii*	0.83
P. jeffreyi	0.16	*P. pinea*	0.08	*A. nordmanniana*	0.62
P. halepensis	0.11	*P. aristata*	0.09	*A. bornmulleriana*	0.71
P. brutia	0.09	*P. edulis*	0.02	*A. alba*	0.81
P. eldarica	0.07	*Tsuga canadensis*	0.52	*A. concolor*	0.73
P. sylvestris	0.11	*T. heterophylla*	0.57	*A. grandis*	0.83
P. resinosa	0.13	*Pseudotsuga menziesii*	1.15	*A. nobilis*	0.87
P. uncinata	0.16	*Picea engelmanii*	0.26	*A. lowiana*	0.75
P. thunbergii	0.12	*P. pungens glauca*	0.21	*Larix leptolepis*	0.38
P. salzmannii	0.13	*P. omorika*	0.17	*L. decidua*	0.42
P. pumilia	0.15	*P. orientalis*	0.22	*L. sibirica*	0.36

Ginkgo biloba is considered to be the most ancient living Gymnosperm fossil on the earth. Based on fossil records, it has existed for *ca.* 200 million years. If one hypothesizes that 14-MHD acid might be an evolutionary marker, then Pinaceae would be phylogenetically closer to *G. biloba* than any other conifer family, which have apparently lost the ability to synthesize 14-MHD acid. However, we are not aware of other dating techniques that could confirm this hypothesis. According to Hierro *et al.* (1), 14-MHD acid accounts for 0.86% of total fatty acids in *G. biloba* seed lipids. Wolff (unpublished data) found independently an even higher value, 1.40%. Takagi and Itabashi (2) found that the acid tentatively identified as 16:2n-6 acid (most probably 14-MHD acid) comprised 0.68% of the neutral lipids, and 1.54% of the polar lipids. Some *Cedrus* and *Abies* species, and *P. menziesii*, have 14-MHD acid contents in this range (Table 1).

ACKNOWLEDGMENT

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Lipid and Fatty Acid Compositions of a Novel Docosahexaenoic Acid-Producing Marine Bacterium

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ABSTRACT: An unidentified bacterial strain, SCRC-21406, isolated from the intestine of a marine fish, *Glossanodon semifasciatus,* produced docosahexaenoic acid at 23% (mol/mol) [= 28% (w/w)] of total fatty acids in a medium containing 0.5% (wt/vol) peptone and 0.1% (wt/vol) yeast extract at 12°C under atmospheric pressure. The cell yield was 0.43 g/L. The major lipids of the strain were phosphatidylethanolamine and phophatidylglycerol. Docosahexaenoic acid was localized at the *sn*-2 positions of both phospholipids. The amounts of polyunsaturated fatty acids other than docosahexaenoic acid were extremely small [<3% (mol/mol)]. Monounsaturated fatty acids of the *cis*-7, *cis*-9 and *cis*-11 types were detected.
Lipids 32, 975–978 (1997).

Docosahexaenoic acid (22:6n-3; DHA) is an essential nutrient for human health judging from its characteristic localization in the body (1), clinical data (2,3), and its physiological role (4–6). Although DHA concentrates are presently produced from fish oil, DHA-producing algae and fungi have been examined as other sources of DHA for a stable and mass production (7–9). On the other hand, there have been few reports of DHA-producing bacteria isolated from deep-sea fishes (10,11). DHA-producing bacteria seem to be useful for not only DHA production but also gene recombination. However, there has been no report of progress regarding these DHA-producing bacteria. One of the reasons seems to be the need for high pressure and a low temperature during cultivation. Recently, we isolated DHA-producing bacteria, which produce a large amount of DHA under atmospheric pressure, from the intestine of *Glossanodon semifasciatus* caught in the Japan Sea near Toyama Bay. One strain of these DHA-producing bacteria, SCRC-21406, was examined with respect to its lipids and fatty acids, and these results are reported here.

EXPERIMENTAL PROCEDURES

Isolation and cultivation of DHA-producing bacteria. Bacterial strains were isolated from the intestines of many marine fishes caught in the Japan Sea near Toyama Bay. Colonies were purified by streaking onto K28 agar comprising 0.5% (wt/vol) peptone, 0.1% (wt/vol) yeast extract, 50% (vol/vol) artificial seawater, and 1.5% (wt/vol) agar in distilled water. SCRC-21406 was isolated from the intestines of *G. semifasciatus,* which lives at 200–500-m depth, in this manner. SCRC-21406 was cultivated in K28 broth with shaking on a rotary shaker at 12°C under atmospheric pressure. The cells were harvested by centrifugation at 1500 × *g* at 4°C for 15 min and then washed with 50% (vol/vol) artificial seawater.

Analysis of lipids and fatty acids. Lipids were extracted from wet cells according to our previous report (12), and identified by thin-layer chromatography and ^{1}H nuclear magnetic resonance (NMR). Fatty acid methyl esters were prepared from the lipids by heating with 7% methanolic HCl solution at 90°C for 1 h, separated on a reverse-phase thin-layer chromatography plate with acetonitrile/water (28:5, vol/vol), and visualized with iodine. They were then examined by gas–liquid chromatography and gas chromatography–mass spectrometry (12). A fatty acid that was identified as DHA was further analyzed by ^{1}H NMR of its methyl ester and by the infrared spectrum of its pyrrolidide derivative (12). ^{1}H NMR analysis was conducted using the same apparatus and the same method as used for the lipids. The infrared spectrum was recorded with a Jasco model FTIR-5300 (Japan Spectroscopic, Tokyo, Japan) using the thin-layer method.

Analysis of the positional distribution of fatty acids in the lipids was carried out using phospholipase A_2 (12).

RESULTS AND DISCUSSION

While the classification of SCRC-21406 remains to be determined, the strain can be excluded from genus *Vibrio* owing to its insensitivity to a vibriostatic compound (13). Therefore, SCRC-21406 seems to differ from other known DHA-producing bacteria (10,11).

The growth curve for SCRC-21406 in this study is shown in Figure 1. After 24 h, the cells had reached 7.3×10^8 c.f.u./mL. At 37 h, the amounts of dry cells, total lipids, and DHA were 0.43 g, 23.4 mg, and 4.2 mg per liter of culture, respectively.

The lipids of SCRC-21406 consisted of phosphatidyl-

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Abbreviations: DHA, docosahexaenoic acid; NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

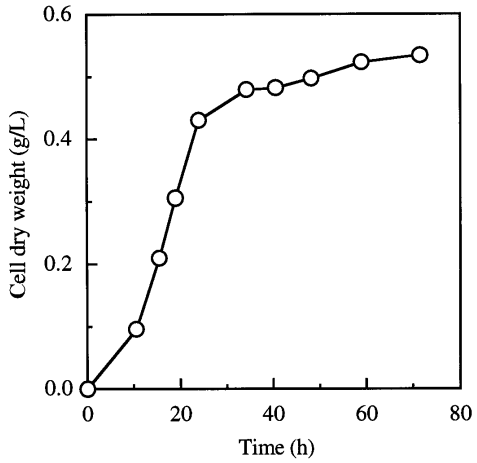

FIG. 1. Growth curve of SCRC-21406 with shaking at 12°C under atmospheric pressure.

ethanolamine (PE) and phosphatidylglycerol (PG) with a mole ratio of 4:1. In the ^{1}H NMR spectrum of the fatty acid methyl ester identified as DHA, the following protons were detected: C*H*$_3$CH$_2$ (0.97 ppm, *t*, 3H), CH$_3$C*H*$_2$ (2.1 ppm, *q*, 2H), C*H*$_2$COOCH$_3$ (2.38 ppm, *m*, 4H), CH=CHC*H*$_2$CH=CH (2.85 ppm, *m*, 10H), COOC*H*$_3$ (3.67 ppm, *s*, 3H), and C*H*=C*H* (5.38 ppm, *m*, 12H). In the mass spectrum of the methyl ester, ions were detected at *m/z* 342 and 311 (Fig. 2). A series of ions at *m/z* 113, 126, 138, 152, 166, 178, 192, 206, 218, 232, 246, 258, 272, 286, 298, 312, 326, 338, 352, 366, and 381 was detected in the mass spectrum of the pyrrolidide derivative (Fig. 3), and their intensities were consistent with those of an authentic sample (data not shown). In the infrared spectrum of the pyrrolidide derivative, since a broad band was detected at 720 cm^{-1} but not over 960–980 cm^{-1} (Fig. 4), the fatty acid has *cis* double bonds but not *trans* ones. These results showed that the fatty acid was *cis*-4, 7, 10, 13, 16, 19-DHA. Other polyunsaturated fatty acids, 18:4n-3, 20:4n-3 and 20:5n-3 (eicosapentaenoic acid), were also identified on the basis of the mass spectra of their pyrrolidide derivatives. Monounsaturated acids, 14:1(*cis*-7), 14:1(*cis*-9), 15:1(*cis*-7), 16:1(*cis*-7), 16:1(*cis*-9), 16:1(*cis*-11), 17:1(*cis*-9), 18:1(*cis*-9), and 18:1(*cis*-11), were identified on the basis of the mass spectra of their trimethylsililoxy derivatives (12). Although the double-bond positions of a 22:5 eluted in front of DHA on gas–liquid chromatography were not determined, it seemed to be of the n-6 type from the relative retention times. The fatty acid compositions of the total lipids, PE, and PG are shown in Table 1. The amounts of polyunsaturated fatty acids other than DHA were extremely small. While the major fatty acid of PE was 16:1(*cis*-9), that of PG was DHA. The positional distributions of the fatty acids in PE and PG are shown in Table 2. In PE and PG, DHA was located at the *sn*-2 position. While 16:1(*cis*-9) was located at the *sn*-2 position in PE, there was more at the *sn*-1 position than at the *sn*-2 position in PG. This indicated that localization at the *sn*-2 position of DHA is more preferential than that of 16:1(*cis*-9). Saturated acids and 16:1(*cis*-7) were localized at the *sn*-1 position in both PE and PG.

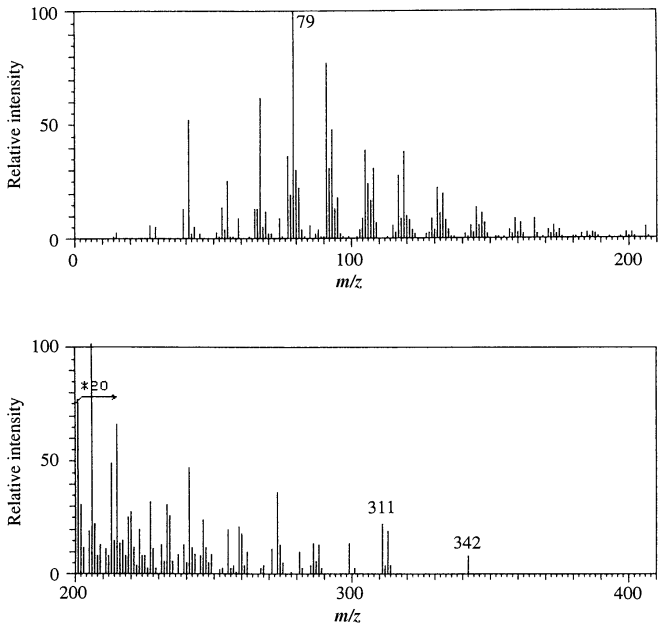

FIG. 2. Mass spectrum of the fatty acid methyl ester identified as docosahexaenoic acid (DHA). The mass spectral analysis was conducted using almost the same method as that described in our previous report (12) except for the column conditions. The column was a capillary one (0.25 m × 25 m) coated with CP-Wax 52 (Chromatopack, Middelburg, The Netherlands). The column temperature was kept at 160°C for the first 1 min, increased to 196°C at 4°C/min, kept for 1 min, increased to 200°C at 4°C/min, kept for 6 min, increased to 232°C at 8°C/min, kept for 3 min, increased to 242°C at 10°C/min, and then maintained for 3 min.

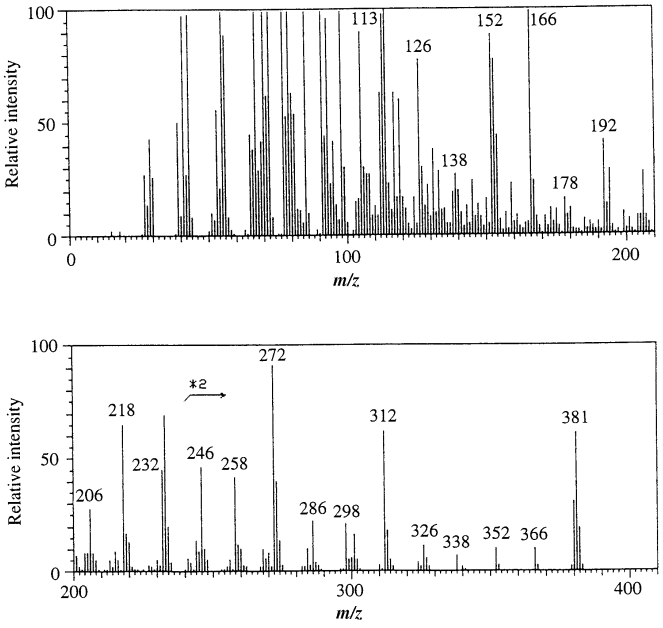

FIG. 3. Mass spectrum of the pyrrolidide derivative identified as DHA. The mass spectral analysis was conducted using the same apparatus and the same conditions as described in our previous report (12). See Figure 2 for abbreviation.

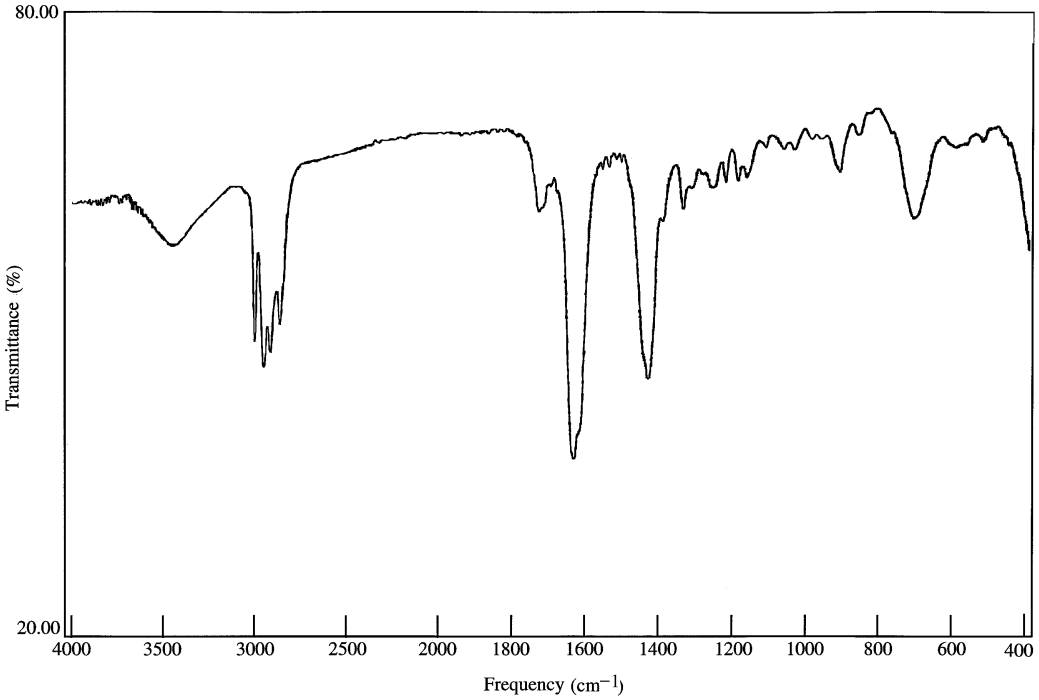

FIG. 4. Infrared spectrum of the pyrrolidide derivative identified as DHA. See Figure 2 for abbreviation.

TABLE 1
Fatty acid Compositions of Total Lipids, PE, and PG of a DHA-Producing Bacterium, SCRC-21406[a]

	Composition (mol%)[b]		
Fatty acids	Total lipids	PE	PG
14:0	15.4 ± 0.3	16.4 ± 0.4	7.9 ± 0.7
14:1(*cis*-7)	3.6 ± 0.1	4.4 ± 0.2	n.d.[c]
14:1(*cis*-9)	0.3 ± 0.0	0.3 ± 0.0	1.0 ± 0.1
iso-15:0[d]	0.4 ± 0.0	0.4 ± 0.0	n.d.
15:0	1.7 ± 0.0	1.6 ± 0.0	1.8 ± 0.1
15:1(*cis*-7)	0.7 ± 0.0	0.8 ± 0.0	n.d.
16:0	13.8 ± 0.1	13.9 ± 0.4	17.6 ± 0.8
16:1(*cis*-7)	7.0 ± 0.3	7.8 ± 0.3	4.3 ± 0.4
16:1(*cis*-9)	29.0 ± 0.3	31.6 ± 0.7	12.5 ± 0.7
16:1(*cis*-11)	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
17:1(*cis*-9)	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.2
18:0	0.4 ± 0.2	0.6 ± 0.3	1.3 ± 0.5
18:1(*cis*-9)	0.6 ± 0.1	0.8 ± 0.4	1.3 ± 0.3
18:1(*cis*-11)	1.0 ± 0.0	0.7 ± 0.0	1.6 ± 0.1
18:4n-3	0.6 ± 0.0	0.6 ± 0.0	0.4 ± 0.0
20:4n-3	0.6 ± 0.0	0.6 ± 0.0	0.8 ± 0.1
20:5n-3	0.8 ± 0.0	0.6 ± 0.0	1.5 ± 0.1
22:5	0.3 ± 0.0	0.3 ± 0.0	0.6 ± 0.0
22:6n-3	23.1 ± 0.6	17.9 ± 0.4	46.9 ± 3.0
Total	100.0	100.0	100.0

[a]Gas–liquid chromatography (GLC) analysis was conducted by almost the same as method described in our previous report (Ref. 12), except for the column temperature. The temperature was kept at 172°C for the first 1 min, increased to 196°C at 4°C/min, kept for 1 min, increased to 200°C at 4°C/min, kept for 6 min, increased to 232°C at 8°C/min, kept for 3 min, increased to 242°C at 10°C/min, and then maintained for 3 min.
[b]The value for each fatty acid is expressed as the mean ± SD; $n = 3$. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DHA, docosahexaenoic acid.
[c]Not detected.
[d]Branched form.

TABLE 2
Positional Distribution of Fatty Acids in PE and PG of a DHA-Producing Bacterium SCRC-21406[a]

	Composition (mol%)[b]			
	PE		PG	
Fatty acids	*sn*-1	*sn*-2	*sn*-1	*sn*-2
14:0	17.4 ± 0.7	0.3 ± 0.1	8.4 ± 0.7	0.4 ± 0.2
14:1(*cis*-7)	1.1 ± 0.1	2.4 ± 0.3	n.d.[c]	n.d.
14:1(*cis*-9)	0.2 ± 0.0	0.1 ± 0.0	n.d.	n.d.
iso-15:0[d]	0.4 ± 0.0	n.d.	1.0 ± 0.1	n.d.
15:0	1.7 ± 0.0	n.d.	2.1 ± 0.0	0.1 ± 0.1
15:1(*cis*-7)	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	n.d.
16:0	14.6 ± 0.2	0.7 ± 0.0	19.2 ± 0.6	1.4 ± 0.5
16:1(*cis*-7)	6.1 ± 0.2	0.4 ± 0.7	4.3 ± 0.1	0.2 ± 0.3
16:1(*cis*-9)	4.7 ± 0.2	28.2 ± 2.4	8.8 ± 0.1	4.3 ± 0.2
16:1(*cis*-11)	0.2 ± 0.0	0.1 ± 0.1	0.3 ± 0.0	n.d.
17:1(*cis*-9)	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
18:0	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.8 ± 0.3
18:1(*cis*-9)	0.3 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.6 ± 0.1
18:1(*cis*-11)	0.5 ± 0.0	0.1 ± 0.0	1.3 ± 0.1	0.2 ± 0.0
18:4n-3	n.d.	0.4 ± 0.1	n.d.	0.3 ± 0.0
20:4n-3	0.4 ± 0.0	0.2 ± 0.0	0.6 ± 0.0	0.2 ± 0.0
20:5n-3	n.d.	0.6 ± 0.0	n.d.	1.3 ± 0.1
22:5	n.d.	0.2 ± 0.0	n.d.	0.4 ± 0.0
22:6n-3	1.6 ± 0.2	14.9 ± 1.8	2.6 ± 0.3	39.7 ± 1.2
Total	50.0	50.0	50.0	50.0

[a]GLC analysis was conducted by the same method as that described in the legend to Table 1.
[b]Mol% was calculated as total percentage of fatty acid of each position to be 50%. The value for each fatty acid is expressed as the mean ± SD; $n = 3$.
[c]Not detected.
[d]Branched form. See Table 1 for abbreviations.

It was proposed that DHA contributes the adaptation to a high hydrostatic pressure and a low temperature, in view of growth condition of DHA-producing bacteria isolated heretofore (10). On the other hand, the phase transition temperature of DHA-linked phosphatidylcholine is higher than that of 16:1(*cis*-9)-linked phosphatidylcholine (14,15). Furthermore, one type of deep-sea bacterium does not produce any polyunsaturated fatty acids and adapts to a high hydrostatic pressure with monounsaturated fatty acids (16). Since SCRC-21406 grows at 12°C under atmospheric pressure, DHA seems to be still more unnecessary for this strain with respect to the improvement of membrane fluidity. There is no information on a bacterial synthetic pathway for DHA. It is suggested that the DHA-synthetic pathway for SCRC-21406 is different from that for mammals which involves one turn of the β-oxidation cycle in the peroxisome (17,18). Since the amount of polyunsaturated fatty acids other than DHA is extremely small in SCRC-21406, the pathway also seems to be different from that hypothesized for fungi (19). The production of various monounsaturated fatty acids for double-bond position, such as 16:1(*cis*-7), 16:1(*cis*-9) and 16:1(*cis*-11), is necessary for three different anaerobic pathways with respect to substrate specificity, three different aerobic desaturases with respect to positional specificity, or a combination of them. Information on the synthetic pathway for monounsaturated fatty acid seems to be necessary for elucidation of the DHA synthetic pathway in SCRC-21406. The physiological role of and synthetic pathway for DHA in the strain are interesting subjects.

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Modulation of Adjuvant-Induced Arthritis by Dietary Arachidonic Acid in Essential Fatty Acid-Deficient Rats

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ABSTRACT: Controlled feeding of linoleic acid (LA) or arachidonic acid (AA) to essential fatty acid-deficient (EFAD) rats was used to define the relationship between dietary AA and the inflammatory response evoked during adjuvant-induced arthritis. Based on energy percentage, EFAD rats were fed AA at the human daily equivalent (1×; 5.5 mg/day) or 10 times that amount (10×; 55 mg/day) or, alternatively, 0.5× of LA (273 mg/day). Feeding of 0.5× LA restored the plasma level of AA to that in chow-fed controls. In contrast, feeding of 1× AA only partially restored the plasma level of AA; 10× AA was required to fully replete AA. In parallel to the degree of repletion of AA in plasma, there were accompanying decreases in the levels of palmitoleic acid, oleic acid, and Mead acid. Compared to rats fed the standard laboratory chow diet (Control), edema in the primary hind footpads was decreased by 87% in EFAD, 71% in EFAD + 1× AA, 45% in EFAD + 10× AA, and 30% in EFAD + 0.5× LA. The decrease in edema in the footpads of EFAD rats was nearly identical to the decrease in edema in the footpads of Control rats dosed with indomethacin. Hind footpad edema correlated with the final AA plasma level and eicosanoid levels extracted from hind footpad tissue, but not with neutrophil infiltration. The data showed that 0.5× LA and 10× AA, but not 1× AA, could quickly replete AA, accompanied by the synthesis of AA-derived eicosanoids and restoration of edema. These results suggest that in humans consumption of the average daily amount of AA without concurrent ingestion of LA would not alleviate an EFAD state.

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Modifications of dietary fatty acids have been shown to exert antiinflammatory effects in both acute and chronic models of inflammation (reviewed, 1–3). Such dietary modifications have included supplementation with fish oils that are enriched with n-3 polyunsaturated fatty acids (reviewed, 4,5) and induction of an essential fatty acid-deficient (EFAD) state by deprivation of n-6 polyunsaturated fatty acids (reviewed, 6–8). Effects have included the amelioration of glomerulonephritis in a murine model of the human disease, systemic lupus erythematosus (9,10), ulcerative colitis (11,12), nephrotoxic nephritis (13,14), myocardial infarction (15,16), psoriasis (17), microcirculatory manifestations of ischemia–reperfusion injury (18), diabetes (19–21), and rheumatoid arthritis (reviewed, 22). In rheumatoid arthritis and other chronic inflammatory diseases, evidence has been accumulating which indicates that the quantity and quality of fat in the diet can affect the etiological course of chronic inflammatory diseases (reviewed, 23). Populations in Western countries consume a relatively high amount of arachidonic acid (AA) (approx. 200 mg/day) and an even higher amount of its precursor, linoleic acid (LA) (approx. 20 g/day), both of which are essential, polyunsaturated fatty acids of the n-6 series (reviewed, 2). Eicosanoid production and ensuing inflammation have been correlated with the amount of AA consumed in the diet (24–26).

Essential fatty acid deficiency has been used as an extreme dietary means to deplete AA in key lipid pools, resulting in an antiinflammatory effect that, in part, is due to decreased synthesis of proinflammatory eicosanoids (reviewed, 7,8). Notable ramifications are decreased synthesis of prostaglandins and leukotrienes, a decreased number of resident macrophages in tissues, and a decreased influx of neutrophils and macrophages into sites of inflammation (27–29). The correlative factor associated with compromised macrophage or neutrophil function appears to be partial depletion and accompanying unavailability of AA in key lipid pools.

Adjuvant-induced arthritis (AIA) in the rat is a chronic inflammatory disease model that has many facets in common with human rheumatoid arthritis (reviewed, 30). As such, the model has been used for several decades as a predictor of clinical efficacy of antiinflammatory drugs. The disease is initiated by the injection of a mycobacterium/mineral oil suspension (complete Freund's adjuvant) into the hind footpad of Lewis rats. Progression of the disease proceeds quickly. Acute inflammation occurs in the primary footpad within 24 h. After a plateau period, severity increases dramatically between days 12–15, at which time symptoms occur in the noninjected, secondary hind footpad. Maximal severity is

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Abbreviations: AA, arachidonic acid; AIA, adjuvant-induced arthritis; bid, twice a day dosing; COX, cyclooxygenase; EFAD, essential fatty acid-deficient; ig, intragastric; INDO, indomethacin; LA, linoleic acid; mpk, milligram per kilogram; MPO, myeloperoxidase; NSAIDs, nonsteroidal antiinflammatory drugs; qd, once a day dosing.

achieved by day 25, while joint deformity occurs by day 35. It is well established that inflammation in AIA is prostaglandin-mediated; nonsteroidal antiinflammatory drugs (NSAIDs) which inhibit cyclooxygenase (COX) activity, notably inducible COX-2 activity, mitigate the inflammatory response, principally owing to inhibition of synthesis of proinflammatory eicosanoids derived from AA (31). The literature is replete with reports on attempts to improve the arthritic index in humans by decreasing the level of AA by dietary means, mainly by fish oil supplementation (reviewed, 22). However, there is a paucity of reports describing the effect that dietary manipulations have on the severity of AIA, especially the effect of dietary AA. Lawrence (32) reported that diets enriched with n-6 polyunsaturated fatty acids exacerbated AIA. Denko (33) reported decreased hind footpad swelling in EFAD rats; edema was restored upon supplemental feeding of corn oil, suggesting a role of n-6 polyunsaturated fatty acids.

The purpose of this study was to examine whether correlations existed in AIA between the level of dietary AA, the ensuing level of plasma AA, and the levels of hind footpad inflammatory markers [i.e., edema, eicosanoids, and myeloperoxidase (MPO) activity]. EFAD rats were utilized because they are severely depleted of both LA and AA. Dietary LA was evaluated along with dietary AA because AA is synthesized from LA *in vivo* by sequential Δ6 desaturation, elongation, and Δ5 desaturation (reviewed, 34). The amount of LA or AA fed to rats was calculated by allometric scaling based on caloric intake from humans to rats, assuming the same intake of fatty acids per kcal per day. Daily feeding of LA (0.5× the human daily equivalent) or AA (1× or 10× the human daily equivalent) to EFAD rats was employed to control repletion of LA and/or AA and to define the relationship between dietary AA and the inflammatory response evoked during AIA. By assuming the validity of allometric scaling based on caloric intake between rodents and humans, the data suggest that, in humans, consumption of the average daily amount of AA would not exacerbate the severity of prostaglandin-mediated inflammation.

MATERIALS AND METHODS

Materials and reagents. Authentic fatty acids and fatty acid methyl esters were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). The vehicle used for oral dosing consisted of 0.5% methylcellulose + 0.025% Tween-20 (polyoxyethylene-sorbital monolaurate), both purchased from Sigma Chemical Co. (St. Louis, MO). The ethyl ester of LA or AA was emulsified in vehicle by sonication and administered orally (0.6 mL/dosing), qd (once a day dosing) (p.m.), starting on day 0 and ending on day 25. Control groups not receiving the fatty acid ethyl esters were gavaged with vehicle only. Indomethacin was purchased from Sigma Chemical Co. and was prepared as a suspension in vehicle by sonication. All solvents and reagents were of analytical grade.

Dietary paradigms and induction of arthritis. Male, Lewis rats were received at 3 wk of age (Harlan Sprague-Dawley, Indianapolis, IN) and were maintained on a a standard rodent diet (Certified Rodent Diet #5002; PMI Feeds Inc., St. Louis, MO), herein referred to as the Control diet, or an EFAD diet (#5803C, low essential fatty acid P.D., Purina Test Diets, Richmond, IN). Water was provided *ad libitum* throughout. The composition of the two diets was as follows:

(i) Control diet (w/w): 55.0% carbohydrates, 20.1% protein, and 4.5% fat (LA, 2.15%; AA, <0.01%). Specific ingredients are ground yellow corn, soybean meal, ground wheat, wheat middlings, fish meal, wheat germ meal, brewer's dried yeast, cane molasses, dried beet pulp, ground oats, alfalfa meal, dried whey, ground soybean hulls, soybean oil, casein, calcium carbonate, dicalcium phosphate, salt, DL-methionine, choline chloride, vitamin A acetate, cholecalciferol, cyanocobalamin, calcium pantothenate, folic acid, riboflavin, thiamine mononitrate, nicotinic acid, pyridoxine hydrochloride, di-alpha tocopheryl acetate, calcium iodate, cobalt carbonate, copper sulfate, manganous oxide, zinc oxide, ferrous carbonate, and zinc sulfate.

(ii) EFAD diet: 21% vitamin-free casein, 69% sucrose, 3% solka floc, 2% PMI vitamin mix, 5% PMI mineral mix #10, 0.15% DL-methionine, and 0.2% choline chloride. Fat, 0.1% (w/w), was composed principally of saturated and monounsaturated fatty acids.

The fatty acid composition of the Control and EFAD diets is shown in Table 1.

Allometric scaling based on caloric intake (percentage energy) was used to calculate the approximate human daily equivalent of LA (545 mg/day) or AA (5.5 mg/day) administered to the rat (Lab Diet™, The Richmond Standard™, *Animal Diet Reference Guide,* PMI Feeds, Inc.) (Table 2).

A state of essential fatty acid deficiency was confirmed by fatty acid composition analysis of plasma; the ratio of Mead acid (20:3n-9)/AA(20:4n-6) was approximately 6 at the end of the study, much higher than the defined minimal value of 0.4 for essential fatty acid deficiency (6). Refer to Scheme 1 for a summary of the testing scheme.

Arthritis was induced on day 0, at which time all of the rats were nearly 13 wk old, having been fed either the Control or EFAD diet for nearly 10 wk. To induce arthritis, the rats were injected with 1 mg of *Mycobacterium butyricum* (Difco Lab-

TABLE 1
Fatty Acid Composition of the Control and EFAD Diets[a]

Fatty acid	Control diet	EFAD diet
16:0	22.8 ± 0.1	40.6 ± 1.3
16:1n-7	4.0 ± 0.1	5.4 ± 0.3
18:0	6.5 ± 0.1	14.3 ± 0.1
18:1n-9	23.6 ± 0.2	36.3 ± 0.9
18:2n-6	35.6 ± 0.1	3.3 ± 0.8
18:3n-3	5.0 ± 0.1	n.d.
20:4n-6	0.2 ± 0.0	n.d.
20:5n-3	1.4 ± 0.0	n.d.
22:6n-3	1.0 ± 0.1	n.d.

[a]n.d., Not detectable (<0.1%). The amounts are expressed as relative percentage (average ± SEM, $n = 3$ replicate samples). EFAD, essential fatty acid-deficient; Control, rats fed standard laboratory chow diet.

		Measure paw edema	
---------->		DAY 0 ---------->	DAY 25
Rats, 21 d of age	Feed EFAD or Control diet for 68 d	Inject mycogen LA/AA supplementation INDOmethacin treatment	Measure: Paw eicosanoids Paw myeloperoxidase (MPO) Plasma fatty acid

SCHEME 1

oratories, Inc., Detroit, MI) in 25 μL of mineral oil (Mallinckrodt, Paris, KY) into the right-hind footpad (35). The rats were sacrificed on day 25, at which time the arthritic response was maximal. At the start of the experiment (day 0), the rats were divided into nine groups. Groups 1–7 were injected with mycogen (n = 10/group). The specific manipulations of each group were as follows: (i) Control: Rats were maintained on a control diet throughout. This "chow" diet has been used routinely to evaluate NSAIDs in this model and yields very reproducible results. The rats were gavaged with vehicle [intragastric (ig), bid (twice a day dosing)] from days 0–25. (ii) Acute EFAD: Rats were fed the control diet for 68 d, after which they were switched to an EFAD diet (day 0) until the end of the experiment (day 25). (iii) INDO: Rats were fed the control diet throughout and dosed orally with indomethacin [1 milligram per kilogram (mpk), ig, bid], starting on day 0. Dosing with indomethacin was maintained until the end of the experiment (day 25). (iv) CHRONIC EFAD (referred to as EFAD): Rats at 21 d of age were maintained on the EFAD diet for 68 d prior to the start of the experiment (day 0). Feeding of the EFAD diet was continued until the end of the experiment (day 25). (v) EFAD + 1× AA: Chronic EFAD rats were gavaged (ig, qd) with 1× AA (5.5 mg/day) from day 0 through day 25. (vi) EFAD + 10× AA: Chronic EFAD rats were gavaged (ig, qd) with 10× AA (55 mg/day) from day 0 through day 25. (vii) EFAD + 0.5× LA: Chronic EFAD rats were gavaged (ig, qd) with 0.5× LA (273 mg/day) from day 0 through day 25. This dosing of LA was chosen because in previous experiments it was found that bolus dosing with 1× LA (545 mg/day) led to a 20–30% mortality. The dosing of LA was therefore halved. Groups 8–9 served as control groups, not being injected with mycogen (n = 6/group). (viii) Control/(−) Mycogen. (ix) EFAD/(−)Mycogen. Results from these negative control groups are not reported because they were either baseline (e.g., edema and prostaglandin levels) or were no different than the mycogen-treated groups (e.g., fatty acid composition).

For all groups, the rats were weighed and both right- and left-hind paw volumes were measured twice weekly during the course of the experiment, starting just before injection on day 0. Footpad volume was determined using a model 7150 plethysmometer (Ugo Basile, Camerio-Varese, Italy). On day 25, blood was collected from unfasted rats by retro-orbital puncture for analysis of plasma fatty acids.

Eicosanoid extraction and quantification. On day 25, the rats were euthanized and five of the secondary (left) footpads from each group were injected with 400 μL of sterile saline containing 10 μM indomethacin (15 mM stock solution in ethanol; Sigma Chemical Co.). The footpads were amputated and subjected to centrifugation (5000 × g) to collect the footpad fluid. The fluid extracted was clear, indicating that it was devoid of blood. Furthermore, in order to prevent any possible clotting of the fluid, four drops of a solution of heparin (Na salt, 10,000 U/mL in saline; Sigma Chemical Co.) were placed at the bottom of each centrifuge tube, and the solution was allowed to dry before beginning centrifugation. Eicosanoid levels of prostaglandin E_2 (PGE_2), thromboxane B_2 (TxB_2), 6-keto-$PGF_{1\alpha}$ (6-keto), leukotriene C_4 (LTC_4), and LTB_4 were quantified directly in the hind-footpad fluid by standard ELISA (Caymen Chemical Co., Ann Arbor, MI). Eicosanoid content is expressed on a whole-footpad basis in order to correlate it to footpad edema.

MPO assay. Amputated hind footpads were frozen in liquid nitrogen, and the paws were then pulverized into small pieces. The pieces were homogenized in phosphate buffer and subjected to centrifugation at 35,000 × g for 20 min. The supernatant was discarded, and the homogenization was repeated in the presence of 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co.), followed by sonication using

TABLE 2
Allometric Scaling Based on Caloric Intake (energy percentage) Used to Calculate Human Daily Equivalent of Linoleic Acid (LA) or Arachidonic Acid (AA) Given to the Rat

	Actual intake		Extrapolated intake (energy percentage)
	Human[a]	Rat[b]	Rat[c]
LA	20 g/day = 10 mg/kcal/day	290 mg/day = 5.3 mg/kcal/day	545 mg/day
AA	200 mg/day = 0.1 mg/kcal/day	<1.0 mg/day = <0.018 mg/kcal/day	5.5 mg/day

[a]Based on estimated amounts from the literature. Approximations: rat: average body weight: 0.25 kg; Consumption of Control diet—by weight: 13.5 g/day (12–15 g/day); by energy: 54.5 kcal/day (based on 55.0% CHO, 20.1% PRO, 4.5% fat (wt%); gross energy = 4.04 kcal/g). Human: average body weight: 70 kg; caloric consumption: 2000 kcal/day.
[b]Based on amount of LA or AA as stated by the manufacturer, assuming an average of 13.5 g diet consumed per day.
[c]Daily consumption of AA equivalent to that of humans (energy percentage). LA: 10 mg/kcal/day × 54.5 kcal/day = 545 mg/day. AA: 0.1 mg/kcal/day × 54.5 kcal/day = 5.5 mg/day.

a W385 ultrasonic processor (Heat Systems-Ultrasonics, Inc., Farmingdale, NY). The samples were subjected to freeze–thaw three times, followed by sonication and centrifugation. The final assay buffer was a mixture of 16.7 mg *o*-dianisidine HCl (Sigma Chemical Co.) per 10 mL distilled water, 1.6 µL of 30% hydrogen peroxide (Sigma Chemical Co.), and 90 mL of 50 mM phosphate buffer (pH 6.0). Seven µL of sample was added to 200 µL of buffer and then analyzed using an EL 340 Biokinetics Reader (Bio-TEK Instruments, Winooski, VT) (450 nM wavelength). Standard curves were made using MPO purified from human neutrophils (Calbiochem, La Jolla, CA).

Fatty acid composition analysis. Plasma fatty acid composition was determined by gas chromatography using electron capture detection. Twenty µL plasma was spiked with 5 µg heneicosanoic acid (21:0; 0.1 mg/mL stock in hexane) and evaporated to dryness under N_2 gas. Lipids were saponified directly by the addition of 1 mL of 2 N KOH/MeOH (4:1) and incubation of the samples in a 60°C water bath for 1 h. Fatty acids were protonated by the addition of 1 mL 88% formic acid and then extracted into hexane by the addition of 1 mL water + 3 mL hexane followed by thorough mixing. The hexane layer was removed and transferred to a new tube containing a small amount of anhydrous Na_2SO_4 to remove any residual water. The hexane layer was transferred to another new tube and evaported to dryness under N_2 gas. For electron capture detection, the fatty acids were derivatized to pentafluorobenzyl esters by the addition of 10 µL diisopropyl ethylamine (Sigma Chemical Co.) + 20 µL 35% pentafluorobenzylbromide in acetonitrile (Pierce Chemical Co., Rockford, IL) in tightly sealed tubes. The samples were incubated for 15 min in a 50°C water bath, after which the contents were evaporated under N_2 gas. One mL hexane was added, and the samples were washed twice with 1 mL water. The hexane layer was removed and evaporated under N_2 gas. The sample residue was resolubilized in 1 mL hexane and then transferred to a gas chromatographic autosampler vial. Derivatized fatty acids were separated and identified using a Hewlett-Packard model 5880 gas chromatograph (Palo Alto, CA) equipped with a fused-silica capillary column (30 m, 0.32 mm i.d., 0.20 µm film thickness; Supelco, Bellefonte, PA), electron capture detector, and an HP-5880A terminal integrator. Fatty acid pentafluorobenzyl esters were identified by comigration with authentic pentafluorobenzyl ester standards. Results are expressed as relative percentage ± SEM.

Statistical analysis. Primary analysis: The F-test with one-way analysis of variance model was first conducted to test whether the mean endpoint values were identical in the nine groups. If significant ($P = 0.05$), the Dunnett's multiple comparison test was then conducted to compare each group with the Control group ($P = 0.05$). Significant differences are indicated by a "*" (Figs. 1–5). Secondary analysis: If there were significant differences in the means between the EFAD and Control groups in the primary analysis, a secondary analysis was conducted to evaluate the effects of LA or AA fed to EFAD rats. The same methods were used as described for the primary analysis, only EFAD served as the control group. Significant differences are indicated by a "†" (Figs. 2–4).

RESULTS

Effect of diet on weight. The initial weights of the rats in the Chow groups (Fig. 1A) were higher than in the EFAD groups (Fig. 1B). Rats in the Control/(–)Mycogen control group gained weight throughout the experiment. Rats in the Control and Acute EFAD groups lost weight during the course of the experiment, owing to the increasing severity of the arthritis. Rats in the INDO group maintained a relatively constant body weight, owing to mitigation of the arthritis by indomethacin. The weights of the rats fed an EFAD diet remained relatively constant during the experimental period.

Effect of diet on plasma fatty acid content. The fatty acid composition of plasma obtained at day 25, the experimental endpoint, is shown for all the groups in Figure 2 (A–E). Mead acid (20:3n-9), the hallmark fatty acid of essential fatty acid deficiency, is synthesized from oleic acid (18:1n-9) as a compensatory fatty acid for AA when mammals are deprived of

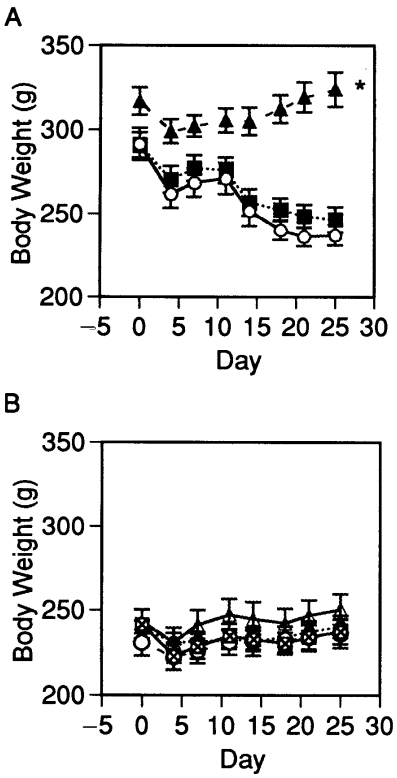

FIG. 1. Body weights of Control (chow groups) (A) or essential fatty acid-deficient (EFAD) (B) groups during the course of adjuvant-induced arthritis. All groups were injected with mycogen on day 0. Body weights (mean ± SEM) were recorded twice weekly for the duration of the experiment. For all groups, $n = 10$, except EFAD + 0.5× LA ($n = 7$). Symbols next to the last data point (day 25) indicate significant differences between the Control (*) or EFAD (†) group ($P < 0.05$). (A) ···■···, Control; —○—, Acute EFAD; -▲--, INDO. (B) ···●···, EFAD; -○--, EFAD + 1× AA; —△—, EFAD + 10× AA; —⊠—, EFAD + 0.5× LA. Abbreviations: INDO, indomethacin; AA, arachidonic acid; LA, linoleic acid.

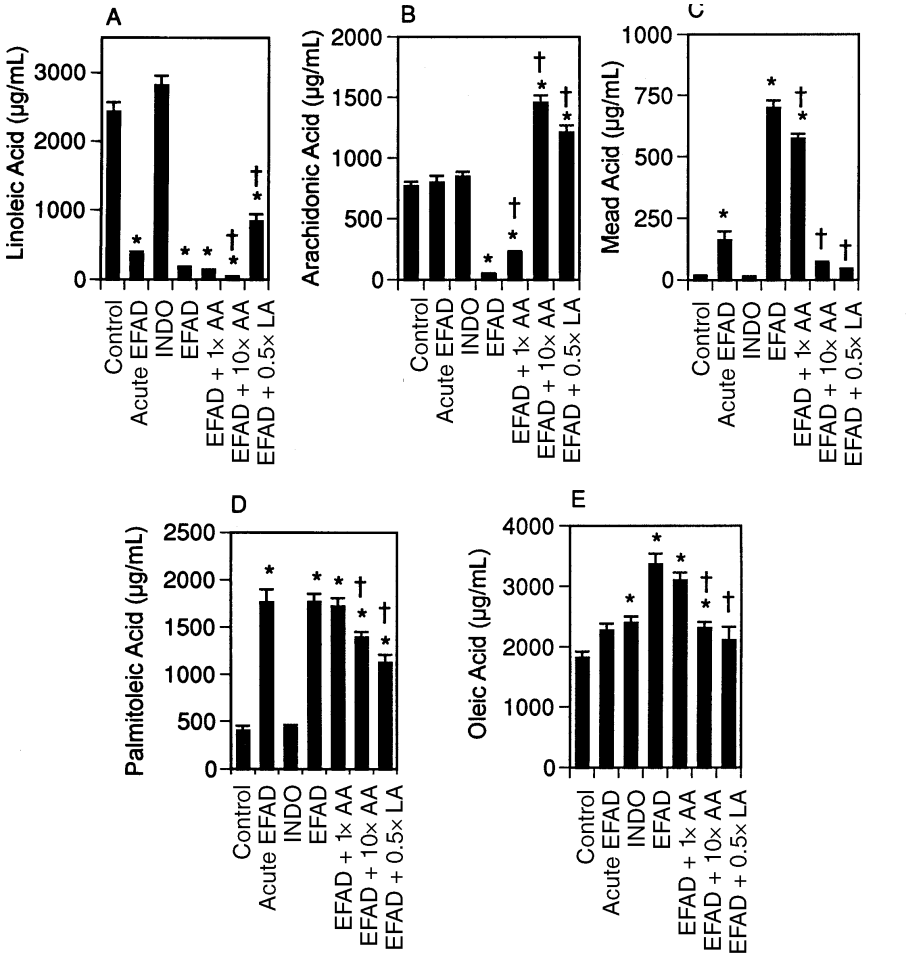

FIG. 2. Plasma fatty acid composition. (A) Linoleic acid (LA) (18:2n-6). (B) Arachidonic acid (AA) (20:4n-6). (C) Mead acid (20:3n-9). (D) Palmitoleic acid (16:1n-7). (E) Oleic acid (18:1n-9). The fatty acid content of plasma was analyzed for each group on day 25, the experimental endpoint. For all groups, $n = 10$, except EFAD + 0.5× LA ($n = 7$). Results are expressed as relative % of total fatty acids (mean ± SEM). Symbols above each bar indicate significant differences between the Control (*) or EFAD (†) group ($P < 0.05$).

n-6 essential fatty acids (reviewed, 6). Palmitoleic (16:1n-7) and oleic acids also accumulate to higher levels in EFAD animals due to induction of the Δ9 desaturase (36).

In the chow-fed groups, the development of arthritis or its mitigation by indomethacin did not cause any alteration in the fatty acid composition; the fatty acid composition remained the same in the Control and INDO groups.

Short-term feeding of the EFAD diet (Acute EFAD) caused changes to occur in some of the fatty acids. LA decreased dramatically (Fig. 2A), whereas AA, the major endproduct of LA metabolism, remained unchanged (Fig. 2B). Mead acid increased (Fig. 2C), as did palmitoleic acid (Fig. 2D) and oleic acid (Fig. 2E).

Chronic feeding of the EFAD diet caused similar changes in fatty acid composition to occur as in Acute EFAD, except that the differences compared to Chow were more extreme; decreases in the n-6 polyunsaturated fatty acids, LA and AA, were compensated for by increases in palmitoleic acid, oleic acid, and Mead acid. The single exception was AA, which was severely depleted in EFAD, but not in Acute EFAD (Fig. 2B). In EFAD rats, the level of AA was only partially restored upon feeding the 1× human daily equivalent of AA (EFAD + 1× AA; Fig. 2B); palmitoleic, oleic, and Mead acids remained relatively high to compensate for the partial depletion of AA. Full repletion of AA occurred only upon feeding the 10× human daily equivalent of AA (EFAD + 10× AA; Fig. 2B); substantive decreases occurred in palmitoleic, oleic, and Mead acids to compensate for the repletion of AA. Feeding the 0.5× human daily equivalent of LA (EFAD + 0.5× LA) gave similar results as did EFAD + 10× AA; there was full repletion of AA and corresponding decreases in the levels of palmitoleic, oleic, and Mead acids. Even though 0.5× LA caused full repletion of AA (Fig. 2B), it caused only partial repletion of LA (Fig. 2A), indicating that LA was quickly metabolized to AA *in vivo*. The saturated fatty acids, stearic (18:0) and palmitic (16:0) acids were unaltered by diet (data not shown).

Effect of diet on hind-footpad edema. The time course of

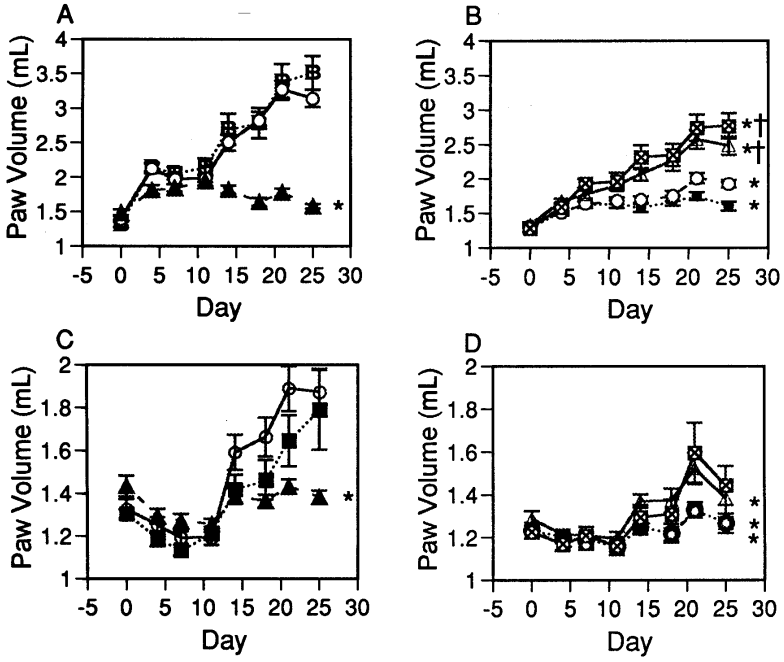

FIG. 3. Time course of mycogen-induced edema in primary and secondary footpads. Mycogen was injected into the right-hind footpads on day 0. Swelling was most apparent in mycogen-injected (primary) footpads, but was also evident in the contralateral, uninjected (secondary) footpads after the second week. For all groups $n = 10$, except EFAD + 0.5× LA ($n = 7$). Results are expressed as paw volume (mL) ± SEM. Symbols next to the last data point (day 25) indicate significant differences between the Control (*) or EFAD (†) group ($P < 0.05$). (A) Control, primary; (B) EFAD, primary; (C) Control, secondary; (D) EFAD, secondary. See Figure 1 for abbreviations. (A) ···■···, Control; —○—, Acute EFAD; -▲-, INDO. (B) ···●···, EFAD; -○-, EFAD + 1× AA; —△—, EFAD + 10× AA; —⊠—, EFAD + 0.5× LA.

mycogen-induced edema is shown for the primary footpads in Figure 3A (Control) and Figure 3B (EFAD) and for the secondary footpads in Figure 3C (Control) and Figure 3D (EFAD). The results were qualitatively the same for the primary and secondary footpads from a given group. The only difference was that the severity of edema was greater in the primary footpads compared to the corresponding secondary footpads. Increases in edema in the primary and secondary footpads in the Control group and mitigation of edema in the INDO group followed the expected progression. Nearly identical increases in edema in the primary and secondary footpads occurred in the Acute EFAD group as in the Control group (Fig. 3A and C), demonstrating that short-term intervention with an EFAD diet was not efficacious. In contrast, edema was mitigated markedly in the chronic EFAD group throughout the time course of the experiment; some initial swelling occurred between days 0–4, after which it plateaued (Fig. 3B and D). On day 25, edema in the EFAD group (Fig. 3B and D) was very similar to that in the INDO group (Fig. 3A and C), demonstrating that EFAD had comparable efficacy as indomethacin. These results are in contrast to the lack of an antiinflammatory response in the Acute EFAD group (Fig. 3A and C). Hence, long-term intervention with an EFAD diet was necessary to evoke an antiinflammatory response.

In the chronic EFAD group, the beneficial effect of EFAD was maintained, even when the rats were dosed with the 1× human daily equivalent of AA (EFAD + 1× AA) (Fig. 3A and B). Compared to the EFAD group, there was perhaps a marginal increase in edema in the EFAD + 1× AA group, but only at the latter time points. In order for dietary AA to evoke a substantial increase in edema, a dosing of the 10× human daily equivalent of AA (EFAD + 10× AA) was necessary (Fig. 3B and D). Significantly, feeding of the 0.5× human daily equivalent of LA was sufficient to increase edema to the same extent as with the 10× human daily equivalent of AA (Fig. 3B and D). It must be kept in mind, however, that the absolute mass of LA at the 0.5× human equivalent (233 mg/day) was still fourfold more than the absolute mass of AA at the 10× human equivalent of AA (57 mg/day). The degree of edema was elevated in animal groups where plasma AA was substantially higher than the EFAD level (i.e., EFAD + 10× AA and EFAD + 0.5× LA).

Effect of diet on hind-footpad eicosanoid levels. The levels of PGE_2, TxB_2, 6-keto-$PGF_{1\alpha}$, and LTC_4 extracted from secondary hind-footpad tissue (Fig. 4A–D) paralleled the level of plasma AA (c.f. Fig. 2B) and the degree of edema (c.f. Fig. 3A–B) in the corresponding Control or EFAD group. LTB_4 was undetectable in any of the control or treatment groups (<20 pg/mL or <2–3 pg/footpad). As predicted from the edema results, eicosanoid levels from the Control and Acute EFAD groups were relatively high. In contrast, the lowest eicosanoid levels were from the INDO and EFAD

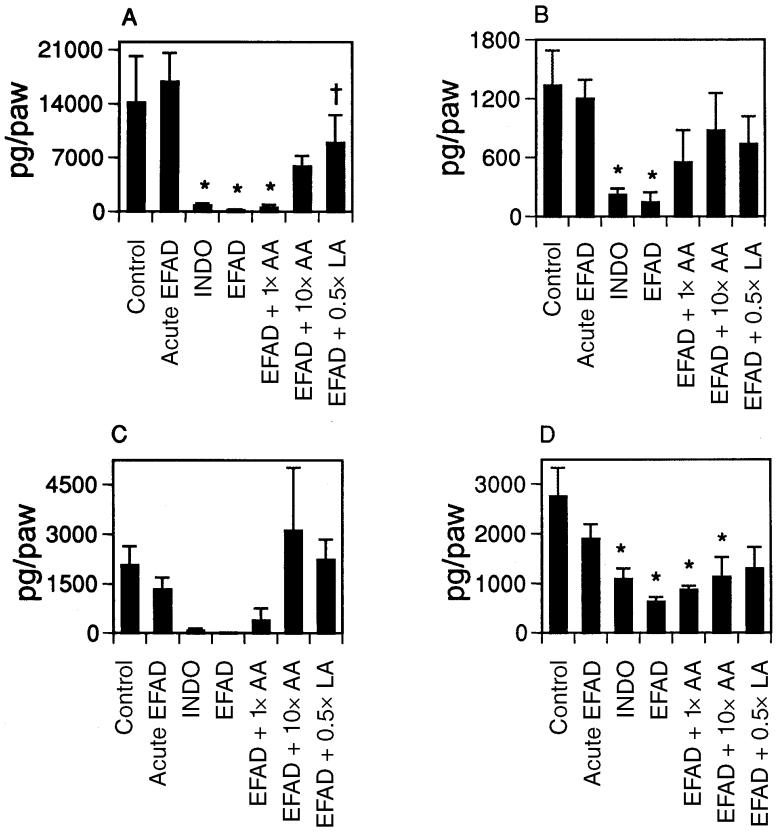

FIG. 4. Eicosanoid levels in hind-footpad extracts. Eicosanoids were quantified by standard ELISA in extracts from secondary hind footpads at day 25, the experimental endpoint. For all groups, $n = 5$, except EFAD + 0.5× LA ($n = 4$). Symbols above each bar indicate significant differences between the Control (*) or EFAD (†) group ($P < 0.05$). (A) PGE_2, (B) TxB_2, (C) 6-keto $PGF_{1\alpha}$, (D) LTC_4. See Figure 1 for abbreviations.

groups. EFAD was just as efficacious in decreasing PGE_2 and 6-keto-$PGF_{1\alpha}$ synthesis as in decreasing edema and was comparable to indomethacin. The antiinflammatory properties of EFAD were also observed in unchallenged rats; there were lower basal levels of PGE_2, TxB_2, and 6-keto-$PGF_{1\alpha}$ in the EFAD/(−)Mycogen group compared to the Control/(−)Mycogen group (data not shown). Dietary supplementation with 1× the human daily equivalent of AA (EFAD + 1× AA) only partially restored eicosanoid levels compared to Control. In order to more fully restore eicosanoid levels, but not to completion, the 10× human daily equivalent of AA (EFAD + 10× AA) or the 0.5× human daily equivalent of LA (EFAD + 0.5× LA) had to be administered.

Effect of diet on neutrophil infiltration. MPO activity, an enzyme marker of neutrophils (37), was quantified in secondary hind-footpad extracts as a measurement of neutrophil infiltration. MPO activity was readily detected and was not greatly affected by diet, even when comparing Control vs. EFAD groups (Fig. 5).

DISCUSSION

AA appears to have a unique preference in mammals compared to other polyunsaturated fatty acids; high-level feeding of AA, but not LA, augmented tissue levels of AA and ensuing eicosanoid production beyond control levels obtained in animals fed a normal chow diet (24,25). The relatively high

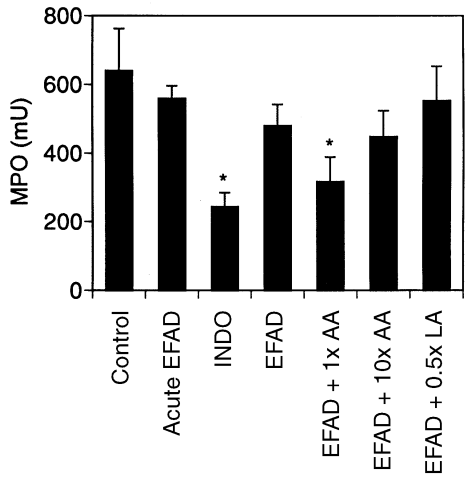

FIG. 5. Myeloperoxidase (MPO) activity in secondary hind-footpad extracts. MPO activity (mU ± SEM) was used as an indicator of neutrophil infiltration at day 25, the experimental endpoint. For all groups, $n = 5$, except EFAD + 0.5× LA ($n = 3$). Symbols above each bar indicate significant differences between the Control (*) or EFAD (†) group ($P < 0.05$).

consumption of n-6 polyunsaturated fatty acids in the Western diet (reviewed, 2), namely, LA (approx. 20 g/day), and, to a much lesser extent, AA (approx. 200 mg/day), raises the possibility that arthritis or other chronic inflammatory diseases could be exacerbated by AA obtained directly in the diet or metabolized *in vivo* from LA. A relatively large clinical effort has attempted to evaluate the role of AA in rheumatoid arthritis, mainly by dietary supplementation with n-3 polyunsaturated fatty acids (20:5n-3 and 22:6n-3) as a means of supplanting existing AA (reviewed, 22). Yet, equivocal results have been obtained in all of these studies, mainly because AA cannot be easily supplanted by 20:5n-3 or 22:6n-3, especially if AA is present in any fish oil-supplemented diet (38,39).

The role of dietary AA in AIA is relevant because proinflammatory eicosanoids derived from AA have been shown to be major contributors of disease severity (31). Essential fatty acid deficiency provides a testable way to evaluate the role of dietary AA in AIA, a rat model of arthritis that mimics many facets of rheumatoid arthritis in humans. Denko (33) showed that EFAD mitigated the severity of arthritis in AIA and that the mitigation was reversed by feeding the animals a corn oil diet. Corn oil is enriched in LA but is totally lacking in AA. Without providing direct evidence, Denko theorized that the LA was metabolized to AA *in vivo* and that proinflammatory eicosanoids derived from AA contributed, in large part, to the inflammatory process. Since EFAD animals are severely depleted of AA, repletion of AA could be titrated precisely by daily feeding of AA. The distribution and metabolism of orally administered AA are similar between EFAD and chow-fed rats (40), lending further validity to the use of the EFAD model as a means to evaluate the effects of dietary AA in AIA. The relevance to the human situation was addressed by feeding rats the daily human equivalent of AA, based on allometric scaling of caloric intake (24). In chronic EFAD rats which were fed either the 1× or 10× human daily equivalent of AA or the 0.5× human daily equivalent of LA, clear correlations were established between the levels of plasma AA (Fig. 2B), hind-footpad edema (Fig. 3B and D), and eicosanoids extracted from edematous hind-footpad tissue (Fig. 4). In contrast, there was no correlation between neutrophil infiltration, as determined by MPO activity, into edematous hind-footpad tissue and the level of plasma AA (Fig. 5). Even though edema and eicosanoid synthesis were markedly decreased in EFAD rats, neutrophil infiltration into secondary hind-footpads occurred unimpeded. This is consistent with data showing that EFAD had no effect on neutrophil infiltration in immune-mediated glomerulonephritis in rats (41).

It was anticipated that acute EFAD would mitigate the severity of arthritis by eliminating AA and its precursor, LA, in the diet. However, acute EFAD did not mitigate edema whatsoever (Fig. 3A and C). Despite the acute EFAD condition, the level of AA remained high in plasma (Fig. 2B) and most likely provided sufficient pools of AA for the synthesis of proinflammatory eicosanoids in hind-footpad tissue (Fig. 4). In contrast to the recalcitrance of AA, rather pronounced changes occurred in other fatty acids during acute EFAD: palmitoleic, oleic, and Mead acids increased, whereas LA decreased. The decrease in LA was striking (Fig. 2A), suggesting that a portion of it was utilized to maintain the level of AA. During the EFAD state, the Δ9 desaturase is induced (36), which would account for the higher levels of palmitoleic, oleic, and Mead acids. These results demonstrated the tenacity with which AA was retained in lipid pools and suggested that long-term curtailment of ingestion of LA or AA might be required to deplete AA sufficiently to manifest an antiinflammatory response. While dietary intervention is probably not feasible, a more acceptable way to decrease AA, besides fish oil supplementation, might be to inhibit Δ6- or Δ5-desaturase activity chronically. Such intervention might result in the antiinflammatory effects associated with EFAD without the accompanying untoward side effects of EFAD (e.g., hair loss, impaired fertility, and psoriatic-type skin) because LA would be present in the diet. Pharmacologic inhibition of the synthesis of AA would allow for LA to be consumed in the diet and, if inhibited at the level of the Δ5-desaturase, for dihomo-γ-linolenic acid (20:3n-6) to serve as the source for PGE_1, thus maintaining gastric and renal function. Also, leukotrienes would not be synthesized from dihomo-γ-linolenic acid because the Δ5 double bond is absent, eliminating another source of proinflammatory mediators. In contrast, no prostaglandins or leukotrienes are synthesized from LA.

The role of dietary AA in modulating the arthritic response in AIA was evaluated by feeding 1× or 10× the human daily equivalent of AA or 0.5× the human daily equivalent of LA to EFAD rats. Since arthritis starts to escalate near day 14, it was reasoned that 2 wk of feeding prior to the arthritic escalation would be sufficient to reach a steady-state level of AA in plasma and inflammatory cells. During the period of maximal arthritic escalation (days 14–25), the AA would then be used as a substrate for the synthesis of proinflammatory eicosanoids. The 1× human daily equivalent of AA was insufficient to restore the plasma level of AA to that of the chow control group (Fig. 2B). In parallel, Mead acid (Fig. 2C), as well as palmitoleic (Fig. 2D) and oleic (Fig. 2E) acids, all products of the Δ9-desaturase, did not decrease in plasma. Based on these results, it was clear why 1× AA was unable to elicit a rebound in edema (Fig. 3) or an increase in eicosanoid production (Fig. 4) in hind footpads. Rather, the 10× human daily equivalent of AA was required to supplant palmitoleic acid (Fig. 2D), oleic acid (Fig. 2E), and, particularly, Mead acid (Fig. 2C) in plasma. Even with full repletion of AA in plasma, there was only a partial rebound in both edema (Fig. 3) and eicosanoid levels in hind footpads (Fig. 4). LA dosed at the 0.5× human daily equivalent was equipotent to 10× AA; AA, synthesized from LA *in vivo* (reviewed, 34), increased to the same level in plasma as in the Control group (Fig. 2B), while palmitoleic (Fig. 2D), oleic (Fig. 2E), and Mead (Fig. 2C) acids decreased. Both edema (Fig. 3) and eicosanoid levels (Fig. 4) rebounded partially in the hind footpads. Even though 0.5× LA resulted in the full repletion of AA in plasma (Fig. 2B), there was only partial repletion of LA in plasma (Fig. 2A). This result, com-

bined with the observation that AA was not depleted during acute EFAD, adds credence to the claim that AA is the most important polyunsaturated fatty acid associated with membrane phospholipids (38).

The combined data suggest strongly that AA obtained directly in the diet or converted from dietary LA by *in vivo* metabolism is an important component of inflammation in AIA; the plasma level of AA (Fig. 2B) correlated with the degree of edema (Fig. 3) and eicosanoid levels extracted from hind footpads (Fig. 4). This would be expected if the progression of AIA depended upon the availability of AA for eicosanoid synthesis. Indeed, prostaglandins derived from AA play a primary role in disease manifestation in AIA (31). Edema and eicosanoid levels were generally highest in the Control and Acute EFAD groups (Figs. 3 and 4). However, the level of plasma AA in those groups was not as high as in the EFAD + 10× AA or EFAD + 0.5× LA groups (Fig. 2B). Thus, while the data indicated that the ready availability of eicosanoid precursor molecules was necessary for disease progression, other factors besides AA, LA, or intermediates between them (i.e., γ-linolenic acid, 18:3n-6, and dihomo-γ-linolenic acid, 20:3n-6) must be involved.

Assuming the validity of allometric scaling based on caloric intake between rodents and humans, the data suggested that in humans the average daily consumption of AA would not exacerbate the severity of prostaglandin-mediated inflammatory diseases. Furthermore, the results suggested that in humans with chronic inflammatory diseases the benefits gained by a low-fat diet would not be compromised by the addition of the average daily amount of AA.

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Preparation of Schiff Base Adducts of Phosphatidylcholine Core Aldehydes and Aminophospholipids, Amino Acids, and Myoglobin[1]

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ABSTRACT: We have prepared Schiff base adducts of the core aldehydes of phosphatidylcholine and aminophospholipids, free amino acids, and myoglobin. The Schiff bases of the ethanolamine and serine glycerophospholipids were obtained by reacting *sn*-1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-glycerophosphocholine (PC-Ald) with a twofold excess of the aminophospholipid in chloroform/methanol 2:1 (vol/vol) for 18 h at room temperature. The Schiff bases of the amino acids and myoglobin were obtained by reacting the aldehyde with an excess of isoleucine, valine, lysine, methyl ester lysine and myoglobin in aqueous methanol for 18 h at room temperature. Prior to isolation, the Schiff bases were reduced with sodium cyanoborohydride in methanol for 30 min at 4°C. The reaction products were characterized by normal-phase high-performance liquid chromatography and on-line mass spectrometry with electrospray ionization. The amino acids and aminophospholipids yielded single adducts. A double adduct was obtained for myoglobin, which theoretically could have accepted up to 23 PC-Ald groups. The yields of the products ranged from 12 to 44% for the aminophospholipids and from 15–57% for the amino acids, while the Schiff base of the myoglobin was estimated at 5% level. The new compounds are used as reference standards for the detection of high molecular weight Schiff bases in lipid extracts of natural products.

Lipids 32, 989–1001 (1997).

The primary products of lipid peroxidation (e.g., hydroperoxides) decompose to form secondary products, which include low and high molecular weight aldehydes (1). The aldehydes may react with cellular components or may be metabolized to inactive tertiary products (alcohols and acids). The low molecular weight bifunctional aldehydes, malonaldehyde and 4-hydroxynonenal, have been extensively investigated for their reactivity with various amines, amino acids and proteins, with which they yield relatively stable covalently bound products. Specifically, malonaldehyde has been shown to undergo 1,4-addition with amino acids to form the eneamines (2), *N*-substituted 3-iminopropenals (3) and *N*,*N*′-disubstituted 1-amino-3-iminopropenes (4). Malonaldehyde also reacts with the amino groups in proteins (4,5) as well as with deoxynucleosides *in vitro* to produce a variety of adducts (6). The early lysine and histidine adduction chemistry of 4-hydroxynonenal has now been elucidated (7,8). It has been shown (7) that a 1:1 Michael adduct predominates in homogeneous aqueous solution and a 1:2 Michael–Schiff base adduct predominates under two-phase aqueous-organic conditions. These findings are in general agreement with recent conclusions regarding the interaction of 4-hydroxynonenal with proteins (9–11). The latter products differ from the amino acid and protein adducts formed with saturated monofunctional lipid ester core aldehydes about to be described. These adducts are more easily reversed than those resulting from the bifunctional aldehydes. Nevertheless, the formation of a Schiff base has been reported between *sn*-1-[9-oxo]nonanyl-2-acetylglycerophosphocholine and bovine thyroglobulin (12), which, following reduction with sodium cyanoborohydride, was suitable for the generation of antibodies that bound specifically to tritiated platelet-activating factor (PAF) and cross-reacted minimally with lyso-PAF, plasmalogens, and other phospholipids. The nature of the amino groups involved in the adduct formation was not established. We have previously identified the lipid ester core aldehydes among the secondary peroxidation products of glycerophospholipids and cholesteryl esters of low density lipoprotein and have noted their partial retention by the apoprotein (13,14). In the following study we have used liquid chromatography/mass spectrometry (LC/MS) with electrospray ionization (ESI) to demonstrate that lipid ester core aldehydes readily form Schiff bases with aminophospholipids, amino acids, and myoglobin, which can be stabilized by reduction with sodium cyanoborohydride before isolation and characterization.

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Abbreviations: GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPS, glycerophosphoserine; HPLC, high-performance liquid chromatography; LC/ESI/MS, liquid chromatography/electrospray ionization/mass spectrometry; PAF, platelet-activating factor; PC, phosphatidylcholine; PC-ALD, 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl GPC; PE, phosphatidylethanolamine; PS, phosphatidylserine; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Materials. Egg yolk phosphatidylcholine (PC), phosphatidylethanolamine (PE), sodium cyanoborohydride, horse skeletal muscle apomyoglobin, MW 16,950 (15), and the amino acids (valine, isoleucine, lysine, and lysine methyl ester) were obtained from Sigma Chemical Co. (St. Louis, MO). Since cyanoborohydride is a potential source of HCN, it should be used in a fume hood and acidification avoided. All solvents were of chromatographic purity, while all chemicals were of reagent grade or better quality and were obtained from local suppliers (Caledon Chemicals, Toronto, Canada).

Preparation of aldehydes. The core aldehydes were prepared from egg yolk PC by ozonolysis and triphenylphosphine reduction as previously described (16). The aldehydes were recovered from the reaction mixture with chloroform and were purified by preparative thin-layer chromatography (TLC) on Silica gel H (250 μm thick layer, 20 × 20-cm glass plate) using a phospholipid solvent system made up of chloroform/ethanol/acetic acid/water 75:45:12:6 (by vol) (16). The aldehyde-containing bands were located by spraying the plate with a Schiff base reagent, which gave a purple color (17). The product (5–10 mg from 10–20 mg egg yolk PC) was made up of 70% 1-palmitoyl-2-[9-oxo]nonanoyl and 30% 1-stearoyl-2-[9-oxo]nonanoyl glycerophosphocholine (GPC).

Preparation of reduced Schiff bases of aminophospholipids. Dioleoyl glycerophosphoethanolamine (GPE) (2 mg) and the 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl GPC (PC-Ald) (1 mg) were dissolved in chloroform/methanol 2:1 and the mixture was kept at room temperature for 1 h. After this time freshly prepared $NaCNBH_3$ in methanol solution was added to a final concentration of 70 mM and the reaction mixture was kept at 4°C for 30 min. At end of reaction, the excess reagent was removed by washing with water. The reduced Schiff base of PE (**I**) was identified by normal-phase LC/ESI/MS as shown below. An identical procedure was used for the preparation and identification of the reduced Schiff base of phosphatidylserine (PS) (**II**). The yields of the Schiff bases were estimated by LC/ESI/MS to range from 30% for the PS adduct to 60% for the PE adduct.

Preparation of reduced Schiff bases of amino acids. The PC-Ald (1 mg) was dissolved in methanol (2 mL), and a twofold molar excess of the amino acid was added as saturated solution in water (2 mL). The reaction mixture was shaken at room temperature for 1 h, and then reduced with $NaCNBH_3$ as described above. The Schiff bases and the residual PC core aldehydes were recovered by extraction with chloroform/methanol 2:1 and were isolated by normal-phase high-performance liquid chromatography (HPLC) and identified by LC/ESI/MS as described below. The yields of the Schiff bases were 15% for lysine (**III**), 30% for isoleucine, 47% for valine (**IV**), and 57% for lysine methyl ester (**V**). Structures **I** to **V** are shown in Scheme 1.

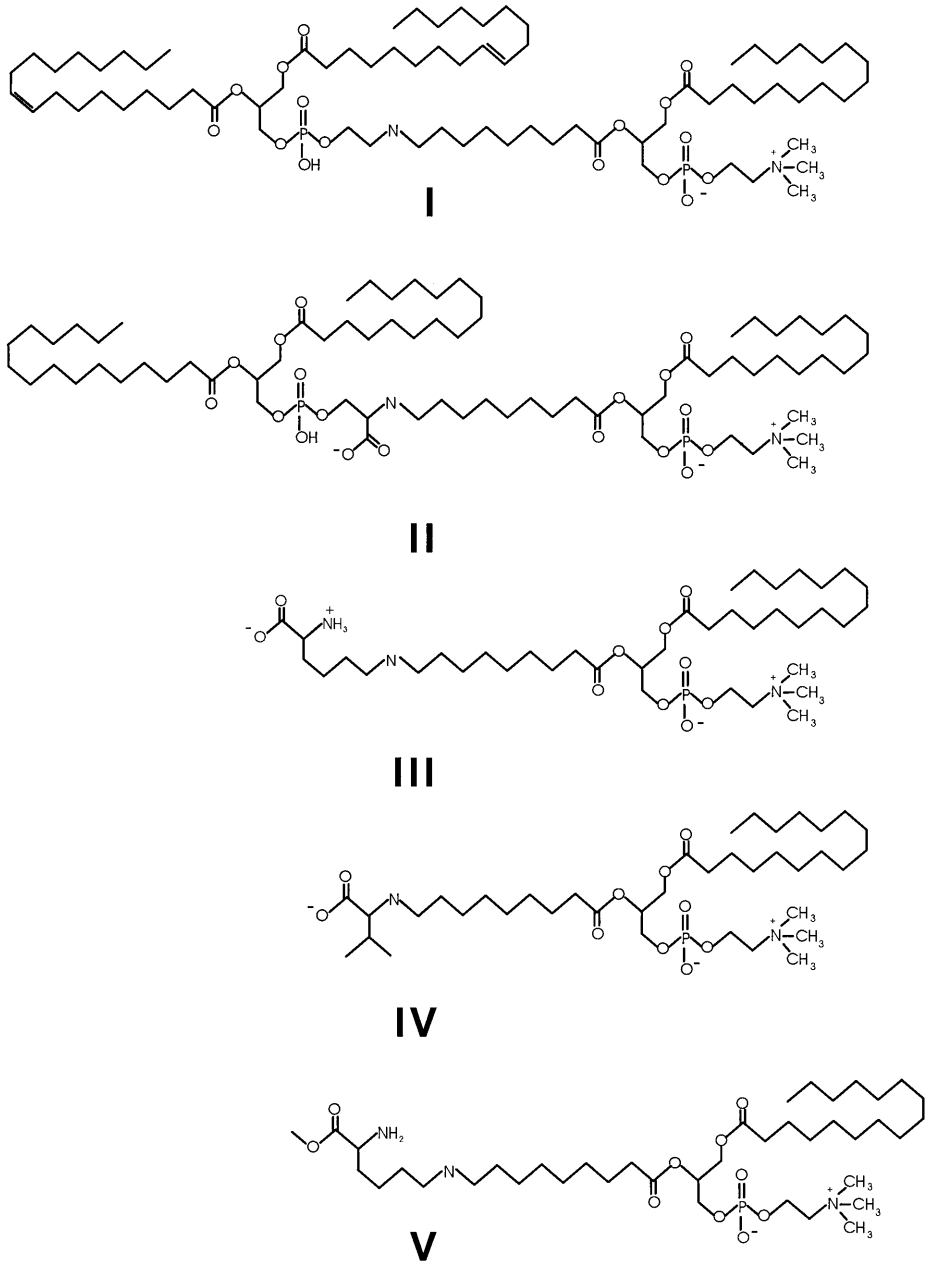
Preparation of reduced Schiff base of the myoglobin . The myoglobin (0.5 mg) was dissolved in distilled water (1 mL). To this solution was added the PC-Ald (2 mg) in ethanol (2 mL) (12) to give an approximate 100:1 ratio of aldehyde to protein. The reaction mixture was kept at room temperature for 1 h. Then $NaCNBH_3$ was added to a final concentration of 70 mM and the mixture kept at 4°C for 30 min. The reaction mixture was dialyzed against distilled water for 24 h with five changes of the solvent in order to remove excess reducing agent. The dialyzed sample was lyophilized and kept at –20°C until further analysis.

HPLC and LC/ESI/MS. Chromatographic analysis of the reduced reaction products of the aminophospholipids or amino acids with PC-Ald were performed on a silica column (Spherisorb, 3 μm, 100 × 4.6 mm i.d., Alltech, Guelph, Ontario) installed in a Hewlett-Packard (Palo Alto, CA) Model 1050 Liquid Chromatograph connected to a Hewlett-Packard Model 5988B quadrupole mass spectrometer equipped with a nebulizer-assisted ESI interface (18). The column was eluted with a linear gradient of 100% A (chloroform/methanol/30% ammonium hydroxide 80:19.5:0.5, by vol) to 100% B (chloroform/methanol/water/30% ammonium hydroxide 60:34:5.5:0.5, by vol) in 14 min, then at 100% B for 10 min (20). Reversed phase LC/ESI/MS was done as described by Kim *et al.* (20). Positive ionization spectra were taken in the *m/z* range 400–1200. Selected-ion mass chromatograms were retrieved from the LC/ESI/MS data. The molecular species of the various Schiff bases were identified from the molecular masses provided by the mass spectrometer, the knowledge of the composition of the reaction mixture, and the relative order of elution (less polar species emerging ahead of more polar species) of the anticipated products from the normal-phase column.

Flow ESI/MS. The Schiff bases of PC-Ald and myoglobin were analyzed by the Hewlett-Packard Model 5988B quadrupole mass spectrometer equipped with the ESI interface using the flow injection mode (19). The lyophilized sample was dissolved in 1 mL methanol/water/acetic acid (50:50:1, by vol), and 50 μL of the sample representing 1.5 nmol protein was injected into the ESI interface at 100 μL/min. Positive ion spectra were taken in the *m/z* range 300–2,000. Similarly the nonreduced Schiff bases of PC-Ald and amino acids were analyzed by flow ESI/MS to indicate that they could be detected as the primary product.

RESULTS

Schiff bases of aminophospholipids. Figure 1 shows the total positive ion current chromatogram (A) of the sodium cyanoborohydride reduced reaction products obtained for dioleoyl GPE along with the reconstructed single-ion chromatograms (B and C) for the $[M + H]^+$ of 16:0-9:0 Ald GPC (*m/z* 1378) and 18:0-9:0 Ald GPC (*m/z* 1406) derivatives, respectively, and the mass spectrum (D) averaged over the entire reduced Schiff base peak (PE + PC Ald). The PE–PC Schiff base is clearly resolved from the unreacted PE and the excess reagent, which has been converted to its hydroxy derivative by the reducing agent, and the small amounts of the azelaoyl GPC and lysoGPC present in the original reagent. On the basis of the peak area proportions, it was estimated



SCHEME 1

that about 40% of the PC-Ald had reacted with the PE. Reconstructed single-ion chromatograms for *m/z* 1378 (B) and 1406 (C), corresponding to $[M + H]^+$ of the reduced Schiff base of the dioleoyl GPE with 16:0-9:0 Ald GPC and 18:0-9:0 Ald GPC, respectively, document coelution of these two products. The mass spectrum averaged over the range of this peak (13.495–14.275 min) shows that the only other high mass ions are due to $[M + Na]^+$ at *m/z* 1400 and 1428, respectively.

Figure 2 shows the fragmentation spectra of the Schiff base of dioleoyl GPE and palmitoyl/[9-oxo]nonanoyl GPC as obtained by increasing the Cap Ex voltage to –300 V in the negative ion mode (A) and to +300 V in the positive ion mode (B). All the major fragment ions detected in the negative and positive ion mode can be assigned to the plausible cleavage products shown in A and B, respectively. The minor ions at *m/z* 125 and *m/z* 86 are due to loss of trimethylamine and phosphoric acid, respectively, from phosphorylcholine (*m/z* 184).

Figure 3 shows the total positive ion current chromatogram (A) of the sodium cyanoborohydride reduced reaction products obtained for dipalmitoyl glycerophosphoserine (GPS) along with the reconstructed single-ion chromatograms (B and C) for 16:0-9:0 Ald GPC (*m/z* 1370) and 18:0-9:0 Ald GPC (*m/z* 1398), respectively, along with the mass spectrum aver-

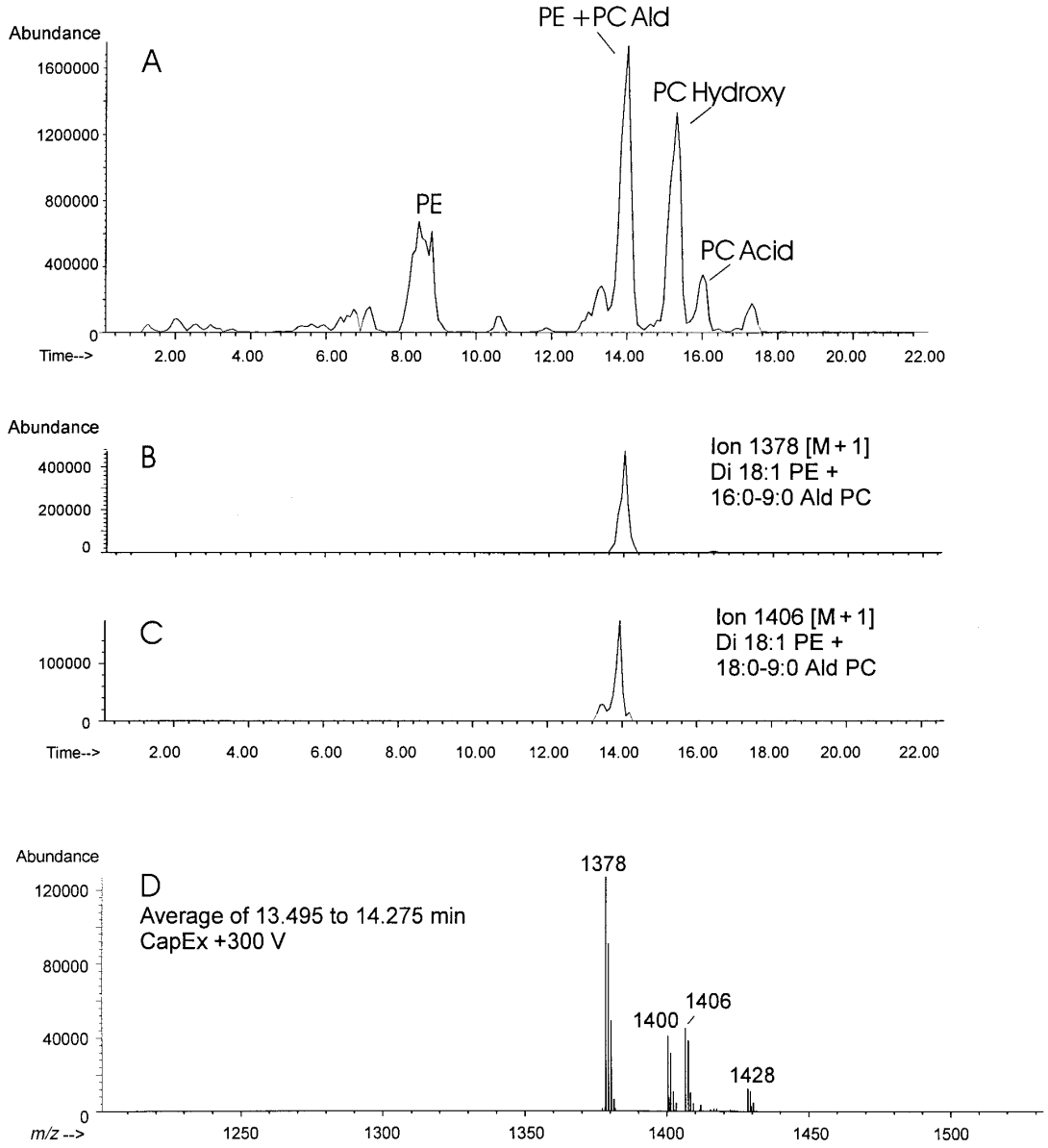


FIG. 1. Normal-phase liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS) of sodium cyanoborohydride-reduced reaction products of dioleoyl glycerophosphoethanolamine (GPE) and 1-palmitoyl(stearoyl)-2-[9-oxo)nonanoyl-*sn*-glycerophosphocholine (GPC). (A), total positive ion current chromatogram; (B) and (C), reconstructed single-ion chromatograms for the 16:0-9:0 Ald GPC (*m/z* 1378) and 16:0-9:0 Ald GPC (*m/z* 1406) derivatives, respectively; (D), total mass spectrum averaged over the entire Schiff base peak in (A). LC/ESI/MS equipment and operating conditions are found in the Materials and Methods section. PE, phosphatidylethanolamine; PC-Ald, phosphatidylcholine core aldehyde; PC Hydroxy, reduction product of PC core aldehyde. Major ions are identified in figure; other ions are described in text.

aged over the entire peak of the Schiff base (D). The reduced PS–PC Schiff base is only partially resolved from the PC 9-hydroxynonanoates [*m/z* 652 and *m/z* 680, not shown in (D)], formed by reduction of the unreacted PC-Ald. The reduced PS–PC Ald Schiff base is preceded by a peak containing a mixture of unidentified PC-Ald condensation products with *m/z* values ranging from 664 to 852. On the basis of the peak area proportions, it was estimated that about 20% of the PC-Ald had reacted with the PS. Reconstructed single-ion chromatograms for the *m/z* 1370 and 1398, corresponding to $[M + H]^+$ of the reduced Schiff base of the dipalmitoyl GPS with 16:0-9:0 Ald GPC and the 18:0-9:0 Ald GPC, respectively, again document coelution of the two products. Only the molecular ions for the PS–PC Ald Schiff bases are seen, with no other ions being detected in the high mass range. Clearly absent are the sodium adducts that were so prominent for the PE–PC Ald Schiff base adducts. The other peaks in Figure 3A were identified as the carboxy (*m/z* 666 and *m/z* 694) and hydroxy (*m/z* 652 and *m/z* 680) derivatives of the 16:0 and 18:0 GPC-Ald. Ionization of the PS–PC Ald Schiff base at Cap Ex of 300 V resulted in fragment ions, which closely resembled the pattern just established for the PE adduct (ion chromatograms not shown).

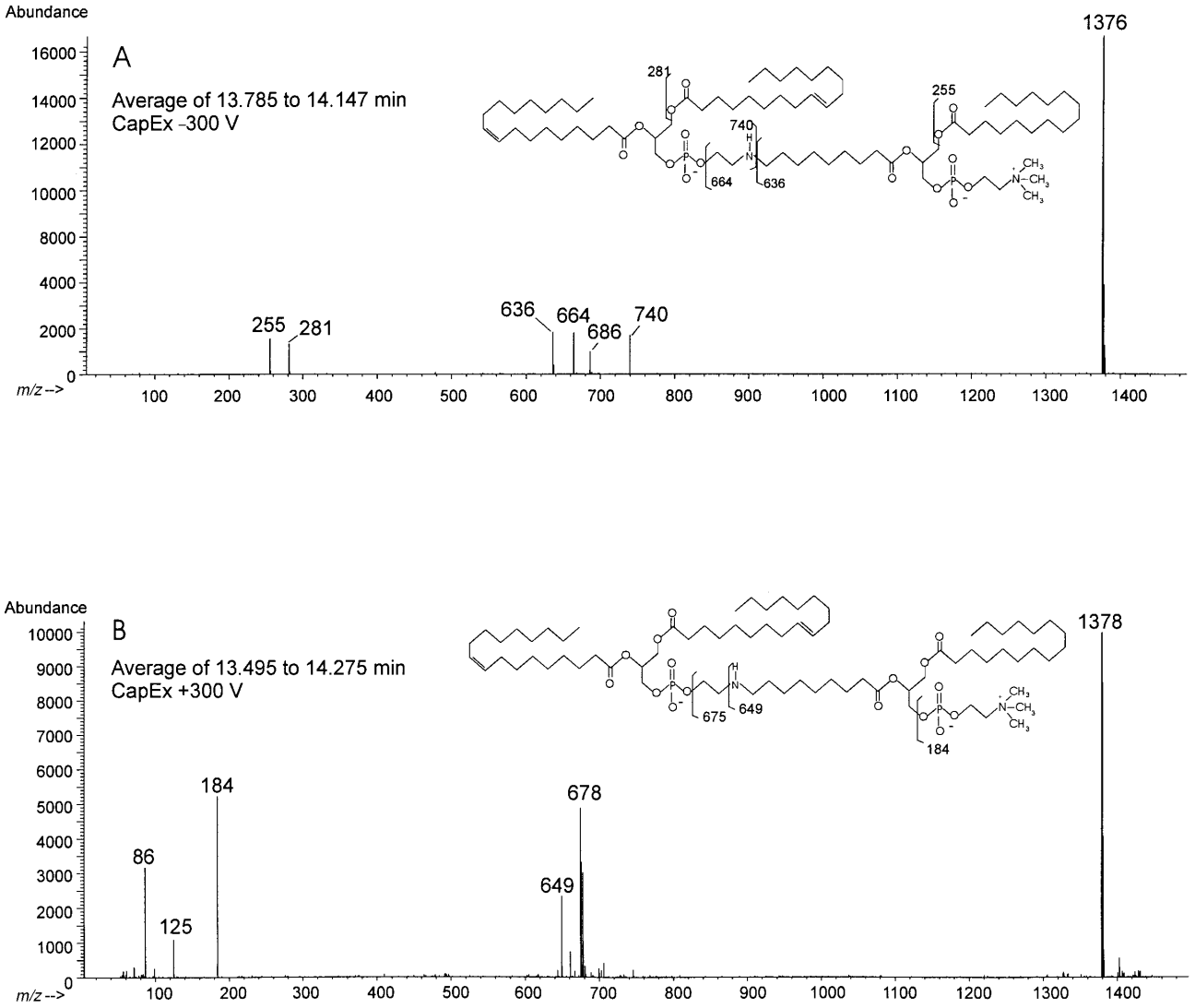


FIG. 2. Normal-phase LC/ESI/MS fragmentation spectra of the reduced Schiff base of dioleoyl GPE and 16:0-9:O Ald GPC at negative Cap Ex voltage of –300 V (A) and of positive Cap Ex voltage of +300 V (B). LC/ESI/MS conditions are as given in Figure 1. The generated ions correspond to the plausible bond cleavages depicted in the sketches accompanying the figures. Other chromatographic and mass spectrometric conditions are as given in the Materials and Methods section. The double bond in the *sn*-1 fatty chains are *cis* and not *trans* as would appear from the artwork. See Figure 1 for abbreviations.

Schiff bases of amino acids. Figure 4 shows the total ion current chromatogram as obtained by reversed-phase LC/ESI/MS for the reduced reaction products of free lysine and 16:0-[9-oxo]nonanoyl GPC (A) and the full mass spectra averaged over the entire peak of the Schiff base (Lys + PC Ald) as obtained by the use of Cap Ex –300 V (B) and Cap Ex +300 V (C), respectively. In both (A) and (B), all the major ions are accounted for by the characteristic fragmentation of the PC moiety indicated in the structural formulae given above. The low molecular weight ion at *m/z* 86 is due to loss of phosphoric acid from phosphocholine, while *m/z* at 71 is due to a cleavage of the α-β carbon bond of bound lysine. The insert in Figure 4A demonstrates that the nonreduced free lysine/PC core aldehyde Schiff base is stable under the conditions of flow injection, yielding the molecular ion (*m/z* 778). The ions at *m/z* 650 and *m/z* 666 are due to the PC acid and aldehyde present in the reaction mixture. It is known that the ε-amino group is more reactive towards Schiff base formation with saturated aldehydes (21).

Figure 5 shows the reconstructed single-ion chromatograms (A and B) corresponding to $[M + H]^+$ ions for the reduced valine-16:0-9:0 Ald GPC (*m/z* 751) and reduced valine-18:0-9:0 Ald GPC (*m/z* 779) adducts, respectively, along with the full mass spectrum (C) averaged over the entire peak of the Schiff base. The mass spectrum shows major ions corresponding to reduced Schiff bases of 16:0-9:0 Ald GPC (*m/z* 751) and of 18:0-9:0 Ald GPC (*m/z* 779) with valine. The ions at *m/z* 773 and *m/z* 801 correspond to the monosodium adducts of the two Schiff bases, respectively.

Figure 6 shows the total positive ion current chromatogram (A) of the reaction mixture of the reduced isoleucine and the

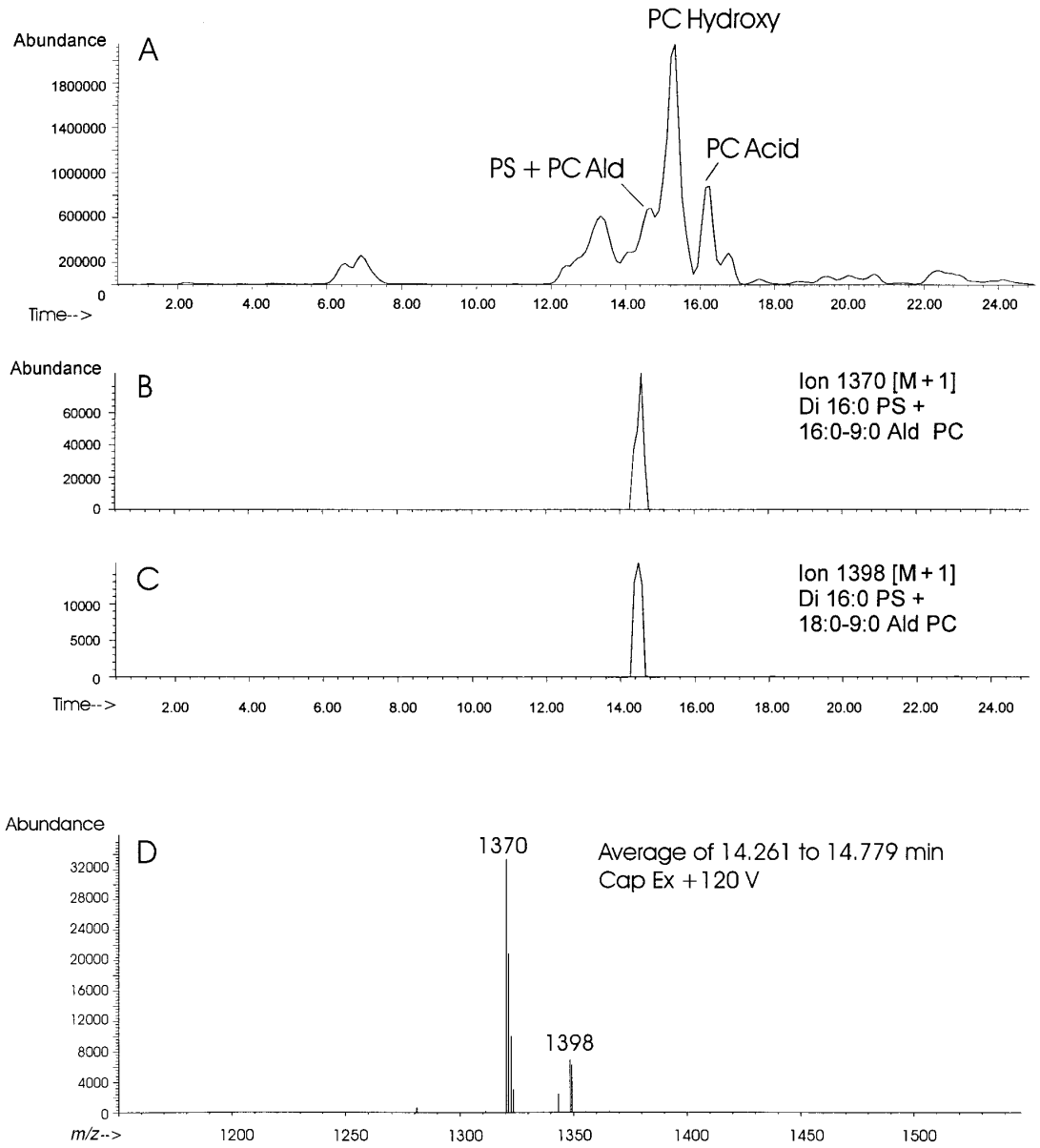


FIG. 3. Normal-phase LC/ESI/MS of sodium cyanoborohydride-reduced reaction products of dipalmitoyl glycerophosphoserine (GPS) and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-*sn*-GPC. (A), total positive ion current chromatogram; (B) and (C), reconstructed single-ion chromatograms for the 16:0-9:0 Ald (*m/z* 1370) and 18:0-9:0 Ald (*m/z* 1398) derivatives, respectively; (D), total mass spectrum averaged over the Schiff base peak in (A). LC/ESI/MS equipment and operating conditions are found in the Materials and Methods section. PS, phosphatidylserine; PC Acid, oxidation product of PC core aldehyde. See Figure 1 for other abbreviations.

PC-Ald along with the reconstructed single-ion chromatograms (B and C) of the isoleucine-16:0-9:0 Ald (*m/z* 765) and isoleucine-18:0-9:0 Ald (*m/z* 793) GPC Schiff base adduct, respectively, with the full mass spectrum (D) averaged over the entire adduct peak (14.464–14.954 min). The mass spectrum of the reduced Schiff base gives major ions corresponding to the adduct of isoleucine and the 16:0-9:0 Ald (*m/z* 765) and the 18:0-9:0 Ald (*m/z* 793) GPC. The ions at *m/z* 787 and *m/z* 815 are due to the monosodium adducts, respectively.

Figure 7 shows the total positive ion current chromatogram (A) of the reaction mixture of the PC-Ald with the methyl ester of lysine along with the single-ion chromatograms (B and C) for the 16:0-9:0 Ald (*m/z* 794) and 18:0-9:0 Ald GPC (*m/z* 822) of the lysine methyl ester Schiff bases, as well as the full mass spectrum (D) averaged over the entire adduct peak. The ions at *m/z* 794 and *m/z* 822 correspond to the reduced Schiff bases of the C_{16} and the C_{18} homologs of the PC-Ald, respectively, while the ions at *m/z* 816 and *m/z* 844 corresponded to the monosodium adducts of the two reduced Schiff bases. The structures of the reduced homologous Schiff bases were confirmed by fragmentation at Cap Ex 300 V.

Schiff bases of myoglobin. Figure 8 shows the flow/

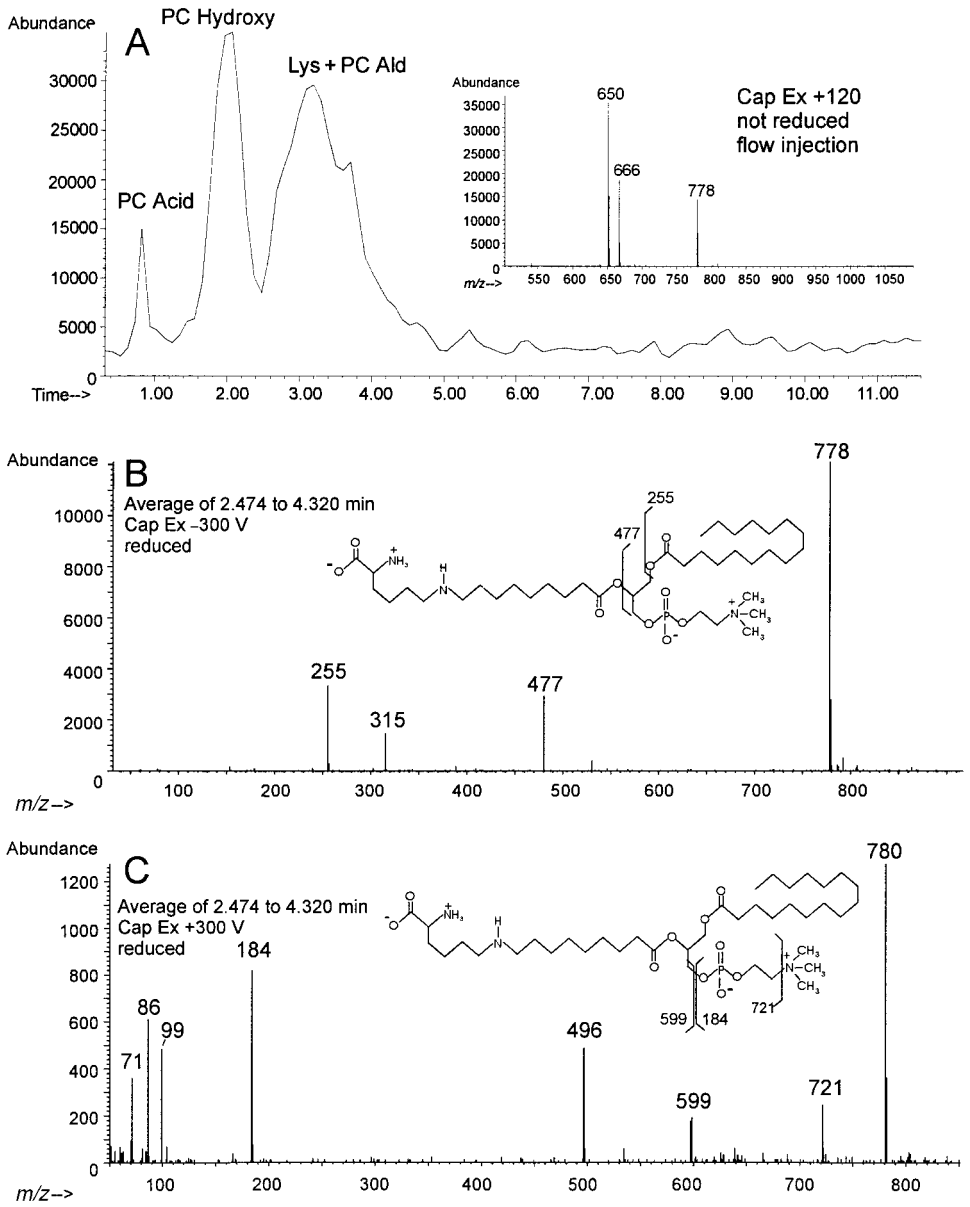


FIG. 4. Reversed phase LC/ESI/MS of the reduced reaction products of free lysine and 16:0/9:0 Ald GPC (A) and fragmentation spectra of the reduced Schiff base of free lysine and 16:0-9:0 Ald PC at negative Cap Ex voltage of –300 V (B) and at positive Cap Ex voltage of +300 V (C). Chromatographic and mass spectrometric conditions are found in the Materials and Methods section. The generated ions correspond to the plausible bond cleavage products depicted in the sketches accompanying the figures. Other ions are as described in text. See Figure 1 for abbreviations.

ESI/MS spectra and the deconvoluted molecular weights of the horse skeletal muscle apomyoglobin (A), its sodium cyanoborohydride reduction product (B), and the product of PC-Ald-apomyoglobin interaction and sodium cyanoborohydride reduction (C). The original apomyoglobin gives a multicharged ion spectrum, which can be deconvoluted to give a MW of 16,948.73. This MW corresponds to the value of 16,950 reported in the literature (17). Following reduction with sodium cyanoborohydride, the apomyoglobin mass spectrum shows two series of multicharged ions, one of which deconvolutes to the original horse skeletal muscle apomyoglobin (major peak) and another one, which deconvolutes to a MW 80 mass units higher (minor peak). The myglobin treated with PC Ald shows the presence of three series of multicharged ions, one corresponding to the original horse skeletal muscle apomyoglobin (MW 16,949.89), a second corresponding to the sodium cyanoborohydride treatment product (MW 17,030.74), and a third corresponding to the reduced PC Ald Schiff base adduct containing two molecules of the 16:0-9:0 Ald (MW 18,218.14). Other incubations gave evidence of the formation of Schiff bases with the mono-16:0-9:0 Ald and mono-18:0-9:0 Ald, and the di-16:0-9:0 Ald and the mixed 16:0-9:0 Ald and 18:0-9:0 Ald adduct. The determination of the actual sites of Schiff base complexing in the apomyoglobin molecules requires further work.

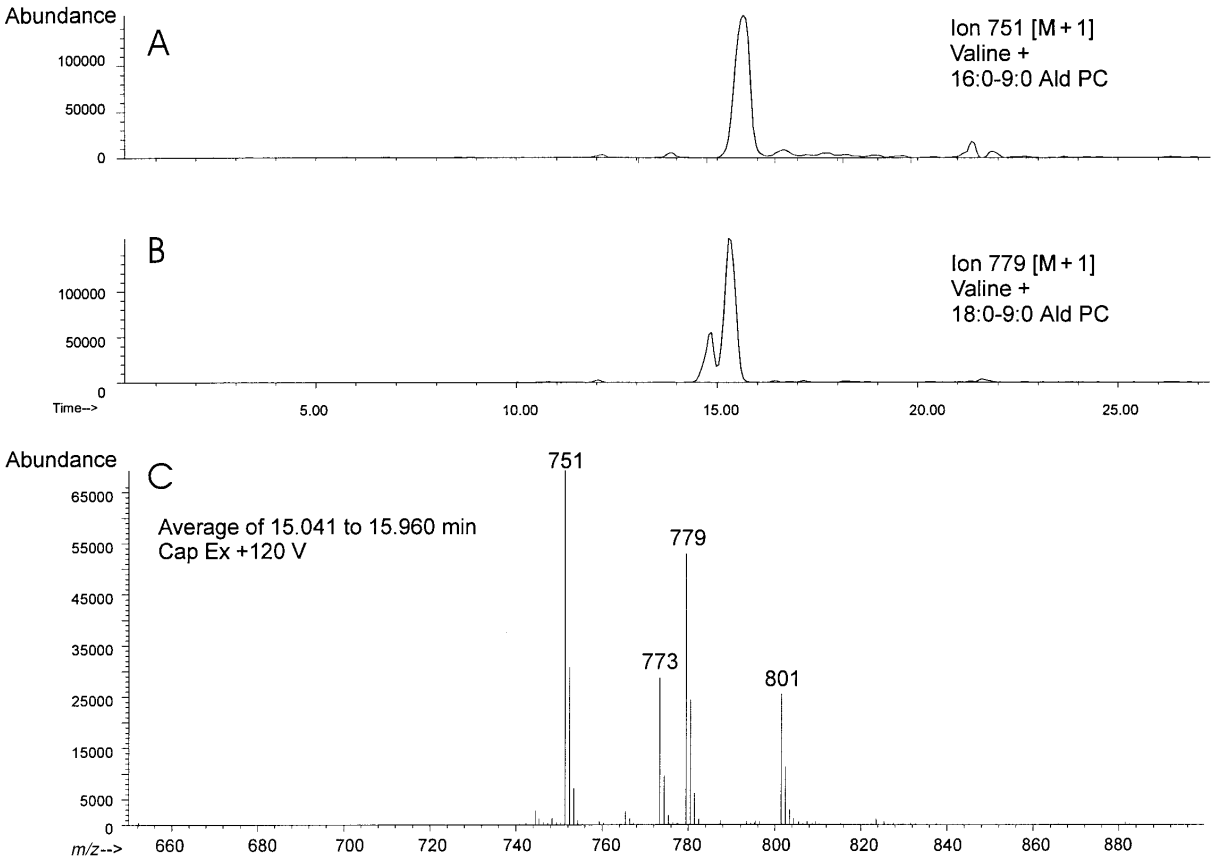


FIG. 5. Normal-phase LC/ESI/MS of sodium cyanoborohydride reduced reaction products of valine and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-*sn*-GPC. (A) and (B), reconstructed single-ion chromatograms for the 16:0-9:0 Ald (*m/z* 751) and 18:0-9:0 Ald (*m/z* 779) derivatives, respectively; (C), total mass spectrum averaged over the entire reduced Schiff base peak. LC/ESI/MS instrumentation and operating conditions are found in the Materials and Methods section. Major ions are identified in figure; other ions are described in text. See Figure 1 for abbreviations.

DISCUSSION

The present study establishes that PC Ald can react with the amino groups of aminophospholipids, amino acids, and polypeptides. Aminophospholipids gave Schiff bases, which could be reduced to yield adducts of characteristic chromatographic and mass spectrometric behavior. There had been no previous demonstration of Schiff base formation between the phospholipid core aldehydes and the amino groups of aminophospholipids. Previous work had shown that the amino groups of the aminophospholipids react with simple aliphatic aldehydes, including malonaldehyde (22), but the exact structure of the products has not been established. Recently, evidence has been obtained for the glucosylation of aminophospholipids (23,24), which represent a type of Schiff base formation. The present study shows that PC Ald also reacts readily with the α-amino group of free amino acids and with the ε-amino group of free and peptide-bound lysine. In a mixed-phase system, the liposomal lipid ester core aldehyde reacts rapidly with the amino compounds to yield a yellow tinge, which deepens with time, resulting in an extensive conversion of the aldehyde into a Schiff's base when incubated with an excess of free amino acid or polypeptide. The Schiff bases could be isolated by TLC and HPLC and could be shown to give molecular ions by flow ESI/MS of concentrated samples in methanol/water solution. This indicated that the Schiff base was the primary reaction product in each instance. Addition of 0.5% ammonia to the HPLC mobile phase resulted in extensive dissociation of the Schiff base and loss of sensitivity of detection. In order to increase the stability of the bases and to permit the use of stronger ionizing solutions, the Schiff bases were reduced with sodium cyanoborohydride. The reduction increased the mass of the Schiff bases by two mass units without any significant effect on their TLC or HPLC migration when compared to the unreduced parent molecules.

Theoretically, free lysine could form Schiff bases either *via* the α- or ε- or both amino groups. Furthermore, the greater reactivity of the primary in comparison to the secondary amino group would suggest that the main product would be the Schiff base of the ε-amino group. This was confirmed by an examination of the Schiff base formed from free lysine and pure 1-palmitoyl 2-[9-oxo]nonanoyl GPC. Both flow injection and reversed-phase LC/ESI/MS with fragmentation of the reduced adduct gave the anticipated ε-amino derivative as the sole or major product. The possibility of for-

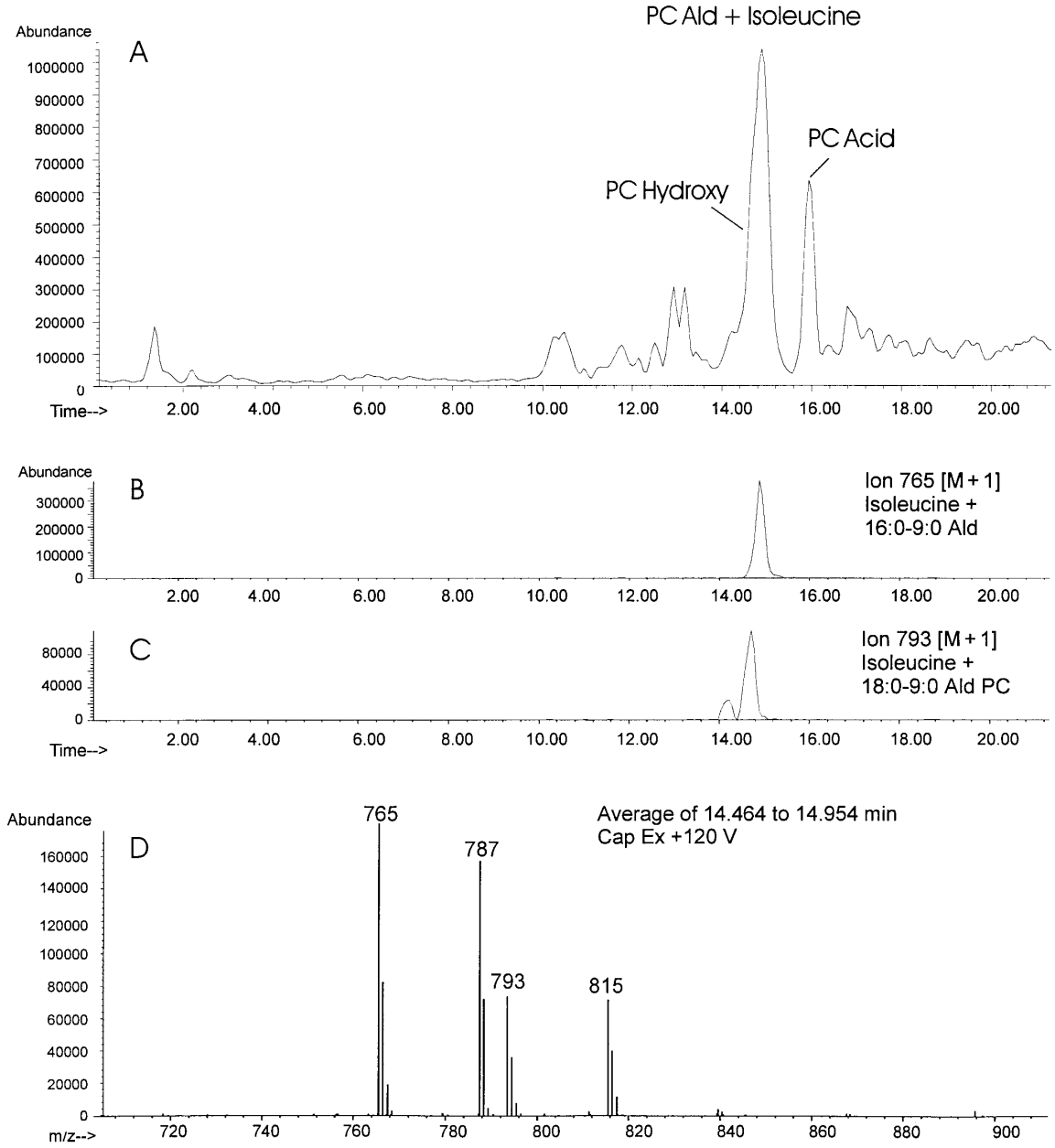


FIG. 6. Normal-phase LC/ESI/MS of sodium cyanoborohydride-reduced reaction products of isoleucine and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-*sn*-GPC. (A), total positive ion current chromatogram; (B) and (C), reconstructed single-ion chromatograms for the 16:0-9:0 Ald (*m/z* 765) and 18:0-9:0 Ald (*m/z* 793) derivatives, respectively; (D), total mass spectrum averaged over the entire reduced Schiff base peak. LC/ESI/MS instrumentation and operating conditions are found in the Materials and Methods section. Major ions are identified in figure; other ions are described in text. See Figure 1 for abbreviations.

mation of an α-amino derivative could not be excluded. The reaction products, if both present, probably would not be separated by the chromatographic methods employed before or after reduction, and would both give the same MW in the mass spectrometer. The matter was not pursued further at this time.

The Schiff bases of the amino acids, including lysine, previously have been prepared using the low MW aldehydes, e.g., malonaldehyde (2-4) and 4-hydroxynonenal (7,8). In these instances, the reaction products are stabilized by secondary reactions, although the structures have not been completely established in all instances. The simple aliphatic aldehydes yield Schiff bases that are more easily dissociated than those of the bifunctional aldehydes and require chemical reduction for stabilization (12,25).

In case of myoglobin, the Schiff base formation would be expected to occur with the N-terminal amino group of glycine and any or all of the ε-amino groups of the internal lysines. Horse skeletal muscle apomyoglobin has a total of 153 amino acid residues with 1 α-amino group and 19 ε-amino groups

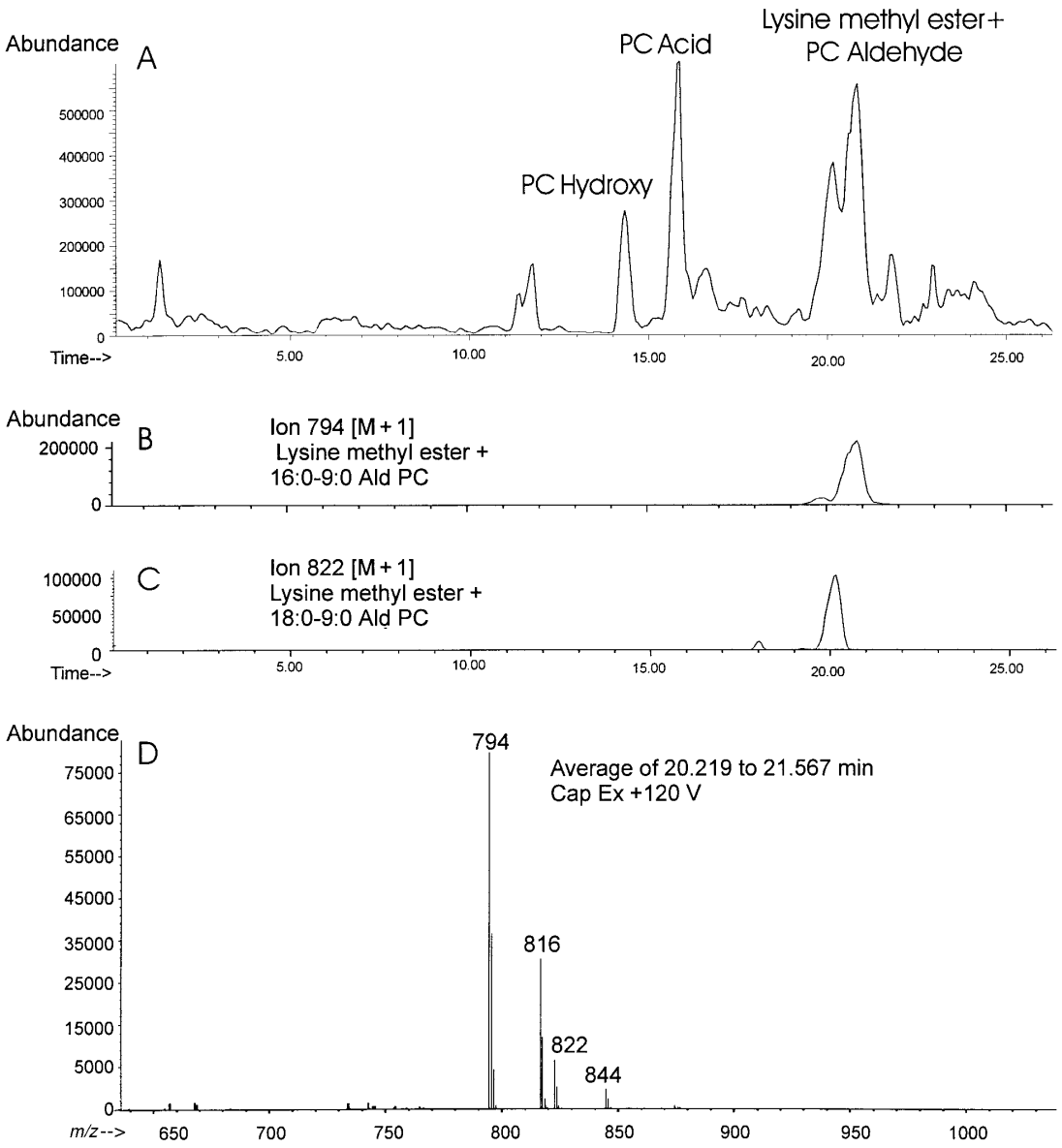


FIG. 7. Normal-phase LC/ESI/MS spectra of sodium cyanoborohydride-reduced reaction products of lysine methyl ester and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-*sn*-GPC. (A), total positive ion current chromatogram; (B) and (C), reconstructed single-ion chromatograms for the 16:0-9:0 Ald (*m/z* 794) and 18:0-9:0 Ald (*m/z* 822) derivatives, respectively; (D), total mass spectrum averaged over the Schiff base peak. LC/ESI/MS instrumentation and operating conditions are found in the Materials and Methods section. Major ions are identified in figure; other ions are described in text. See Figure 1 for abbreviations.

that can react with carbonyl compounds. In the present experiments, however, only two and no more than four PC Ald appeared to be involved in Schiff base formation with horse skeletal muscle apomyoglobin. It is possible that the apomyoglobin molecule contains two particularly reactive sites susceptible to Schiff base formation, which could explain the presence of only minor amounts of the single Schiff base adduct. The formation of a bis-Schiff base adduct corresponding only to the 1-palmitoyl species is probably due to the predominance of this species (80%) compared to the 1-stearoyl species (20%) in the reaction mixture. The reduced bis-Schiff base adduct of the dipalmitoyl derivative was selected for illustration.

In other studies LC/MS with electrospray has been employed to demonstrate the Schiff base formation between acetone and the ε-amino groups of horse myoglobin lysine (26). The identified protein species contained from 1 to 6 adducts of methyl isobutyl ketone or acetone. In the present experiments, two to four residues of the core aldehyde were bound to horse apomyoglobin. The specific amino acid residues involved in the Schiff base formation were not determined. Since the amino acid sequence of horse myoglobin is known, the exact location of the Schiff base forming lysines could be established in the future by trypsin digestion and reversed-phase LC/ESI/MS of the released peptides, the MW of which

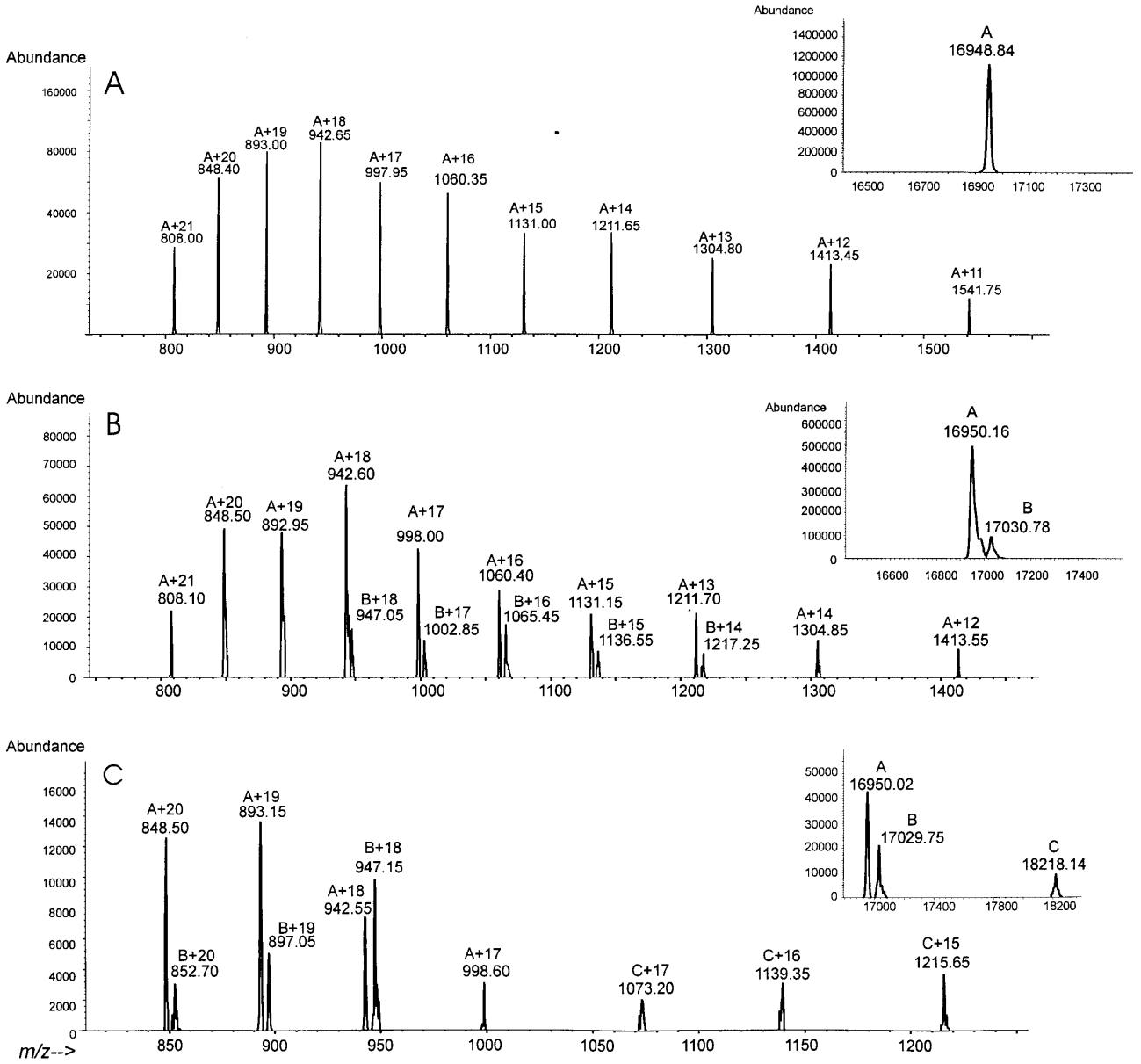


FIG. 8. Flow ESI/MS spectra and the deconvoluted MW of the horse skeletal muscle apomyoglobin (MW 16,948.84) (A), its sodium cyanoborohydride reduction product (MW 17,030.78) (B), and the product of interaction of myoglobin with two 16:0-9:0 Ald GPC (MW 18,218.14) (C). Flow injection ES/MS instrumentation and operating conditions are found in the Materials and Methods section. Major ions and charge distribution are given in figure. See Figure 1 for abbreviations.

could be calculated. The peptides bearing the phospholipid moieties would be expected to be retained much longer on reversed-phase columns than the corresponding peptides without the phospholipid moiety (8).

The previously prepared Schiff base of C_9 core aldehyde of 2-acetylglycerophosphocholine and thyroglobulin (12) was not characterized beyond the demonstration that an antibody could be raised to the reduced adduct to recognize in tissue extracts the PAF, which the C_9 core aldehyde resembles structurally. Uncharacterized have also remained the radioactive complexes formed between apoprotein B and oxidized 2-[1-^{14}C]arachidonoyl PC (27), which must have involved the C_5 core aldehyde, because the malonaldehyde and 4-hydroxynonenal would not be labeled.

The present findings are of interest because the lipid ester core aldehydes like the short-chain aldehydes would be expected to form covalently-bound complexes with proteins. Malonaldehyde and 4-hydroxynonenal-modified lipoproteins have been found entrapped in aortic walls of both humans and animals (28,29), while lysine modification of low density lipoprotein or lipoprotein (a) by 4-hydroxynonenal or malonaldehyde decreases platelet serotonin secretion (30). The generation of reactive aldehyde species next to membrane components possessing active amino groups, which may lead to

Schiff base formation, could lead to membrane damage, loss of enzyme activity, and protein–protein or protein–lipid cross-linking (31). Proteins in the red blood cell membrane show increase in MW, cross-linking, when exposed to lipid-oxidizing conditions (32). The hydrophobicity acquired from complexing with the lipid ester core aldehydes would promote greatly the membrane association of the proteins and contribute to their resistance to proteolytic digestion and *trans*-membrane transport among other effects. Recent work (33) with monoclonal antiphospholipid antibodies has shown that they are directed against epitopes of oxidized phospholipids. A variety of structures that could occur in peroxidized tissues have been suggested, but only a few have been experimentally demonstrated. The present work shows that some of these compounds can be synthesized as well as records the LC/ESI/MS characteristics of these compounds that will be required for the isolation and identification of the Schiff base adducts from natural sources.

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Analysis of Oxylipins by High-Performance Liquid Chromatography with Evaporative Light-Scattering Detection and Particle Beam–Mass Spectrometry

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ABSTRACT: The metabolism of 13*S*-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid was investigated in a crude enzyme extract from mung bean seedlings (*Phaseolus radiatus* L.). Hydroperoxide-metabolizing activity was mainly due to a hydroperoxide lyase and, to a lesser extent, to an allene oxide synthase and a peroxygenase. Oxylipins originating from hydrolysis and cyclization of the allene oxide synthase product 12,13-epoxy-9*Z*,11,15*Z*-octadecatrienoic acid and from peroxygenase catalysis were identified by high-performance liquid chromatography (HPLC) particle beam–mass spectrometry (PB-MS) and quantified by normal-phase HPLC with an evaporative light-scattering detector (ELSD). An advantage of this methodology was the possibility to avoid extensive derivatization procedures commonly used for the gas chromatographic analysis of oxylipins. Owing to a comparable sample inlet system, the ELSD served an important analytical pilot function for the PB–MS: Qualitatively identical chromatographic patterns were obtained with both detection systems. The HPLC system enabled the separation of methyl 12-oxo-phytodienoate, methyl 11-hydroxy-12-oxo-9*Z*,15*Z*-octadecadienoate, methyl 12-oxo-13-hydroxy-9*Z*,15*Z*-octadecadienoate, methyl 9-hydroxy-12-oxo-10*E*,15*Z*-octadecadienoate, methyl 13-hydroxy-9*Z*,11*E*,15*Z*-octadecatrienoate, methyl 15,16-epoxy-13-hydroxy-9*Z*,11*E*-octadecadienoate, and methyl 13-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoate on a Lichrospher DIOL column within 33 min. Compared with a diode array detector, the ELSD proved to be more sensitive, in the case of methyl 12-oxo-13-hydroxy-9*Z*,15*Z*-octadecadienoate by a factor of about 15. In addition, volatile metabolites were analyzed by capillary gas chromatography. The yield of the hydroperoxide lyase product 2*E*-hexenal was 49%, whereas the sum of oxylipins reached about 15%.

Lipids 32, 1003–1010 (1997).

Plant allene oxide synthases are cytochrome P450 enzymes which convert the 13*S*-hydroperoxides of linoleic or linolenic acid into allene oxides (1). Hamberg (2) was the first to demonstrate the formation of the allene oxide 12,13-epoxy-9*Z*,11-octadecadienoic acid (12,13-EOD) by the incubation of 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13-HPOD) with an allene oxide synthase from corn. Structural elucidation of 12,13-epoxy-9*Z*,11,15*Z*-octadecatrienoic acid (12,13-EOT) derived from 13*S*-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid (13-HPOT) has been reported by Brash and co-workers using an enzyme preparation from flaxseed (3). Allene oxides are extremely unstable metabolites possessing a chemical half-life on the order of 15–33 s at 0°C. In aqueous medium they are spontaneously hydrolyzed into ketol derivatives and cyclopentenone fatty acids (2,3). Hydrolysis of 12,13-EOT leads to the formation of the oxylipins 12-oxo-13-hydroxy-9*Z*,15*Z*-octadecadienoic acid (12,13-α-ketol), 9-hydroxy-12-oxo-10*E*,15*Z*-octadecadienoic acid (9,12-γ-ketol), and racemic *cis*-12-oxo-phytodienoic acid (12-oxo-PDA). Enzymatic cyclization of 12,13-EOT, which can occur in the presence of an allene oxide cyclase, results in the formation of the 9*S*,13*S*-enantiomer of 12-oxo-PDA (4), a precursor of the plant growth hormone jasmonic acid (5).

Analysis of these oxylipins is hampered by at least two factors: first, their structural instability when they are exposed to elevated temperatures (such as under gas chromatographic conditions), and second, the commercial unavailability of reference substances, except for 12-oxo-PDA. In recent literature, several methods have been described to quantify the products generated during allene oxide synthase catalysis. One method employed gas chromatographic analysis after multiple derivatization such as methylation, hydrogenation, and trimethylsilylation (6). For improved analysis of the labile ketol this derivatization procedure was necessary; nevertheless, it is time-consuming, and quantitative derivatization at each step is prerequisite for obtaining accurate data. A second method implied the use of a radiolabeled precursor, namely [1-^{14}C]13-HPOT. The composition of labeled prod-

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Abbreviations: CI, chemical ionization; DAD, diode array detector; 15,16-EHOD, 15,16-epoxy-13-hydroxy-9*Z*,11*E*-octadecadienoic acid; EI, electron impact; ELSD, evaporative light-scattering detector; 12,13-EOD, 12,13-epoxy-9*Z*,11-octadecadienoic acid; 12,13-EOT, 12,13-epoxy-9*Z*,11,15*Z*-octadecatrienoic acid; GC–MS, gas chromatography–mass spectrometry; 13-HOD, 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid; 13-HOT, 13*S*-hydroxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid; HPLC, high-performance liquid chromatography; 13-HPOD, 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; 13-HPOT, 13*S*-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid; 11,12-α-ketol, 11-hydroxy-12-oxo-9*Z*,15*Z*-octadecadienoic acid; 12,13-α-ketol, 12-oxo-13-hydroxy-9*Z*,15*Z*-octadecadienoic acid; 9,12-γ-ketol, 9-hydroxy-12-oxo-10*E*,15*Z*-octadecadienoic acid; LC–MS, liquid chromatography–mass spectrometry; 12-oxo-DDA, 12-oxo-10*E*-dodecenoic acid; 12-oxo-PDA, 12-oxo-phytodienoic acid; PB–MS, particle beam–mass spectrometry.

ucts was estimated either by thin-layer chromatography using a scintillation scanner (4) or by high-performance liquid chromatography (HPLC) equipped with a scintillation detector (7,8). However, subsequent derivatization and gas chromatography–mass spectrometry (GC–MS) analysis of collected peaks or spots are required for identification of the metabolites in both cases. In a third method, allene oxide synthase activity was assayed by the spectrophotometric measurement of the decrease in absorbance at 235 nm (9). This decrease is caused by the degradation of the conjugated diene chromophore in the hydroperoxy fatty acid leading *via* the allene oxide mainly to the α-ketol, which shows no absorbance at this wavelength. A major drawback of this assay is its nonspecificity. It does not differentiate between allene oxide synthase and other hydroperoxide-metabolizing enzymes, e.g., hydroperoxide lyases (reviewed in References 10–12), nor is any information provided about the ratio of the products.

As to the identification of labile compounds, liquid chromatography–mass spectrometry (LC–MS) techniques are of increasing importance. Fast-atom bombardment and negative ion electrospray ionization low-energy tandem mass spectrometry have been used for the analysis of polyhydroxy-substituted eicosanoids (13,14) and unsaturated monohydroxy fatty acids (15). Monohydroxy-epoxy derivatives of arachidonic acid (hepoxilins) as well as mono/polyhydroxy-substituted eicosanoids have been investigated after preparation of an anthryl ester derivative by thermospray ionization mass spectrometry (16).

The present study reports on an alternative method for the analysis of oxylipins which overcomes tedious derivatization procedures commonly used for GC analysis. Quantification and identification of oxylipins were performed using a normal-phase HPLC system coupled to an evaporative light-scattering detector (ELSD) and by LC–MS with a particle beam interface. This analytical system was applied to monitor the product spectrum from the metabolism of 13-HPOT by mung bean seedlings, which has not yet been investigated comprehensively.

MATERIALS AND METHODS

Materials. Mung beans (*Phaseolus radiatus* L.) were obtained from a local market. Linolenic acid (99%) and 2-propanol (HPLC-grade) were purchased from Fluka (Neu-Ulm, Germany). Lipoxygenase (EC 1.13.11.12) from soybean type 1-B (110,600 units/mg solid) and methyl ricinoleate (99%) were from Sigma (Deisenhofen, Germany). *n*-Hexane (HPLC-grade) was from Roth (Karlsruhe, Germany).

Preparation of 13-HPOT. About 150 mg linolenic acid was emulsified in 80 mL 0.1 M K_2HPO_4 buffer pH 9.0 using an ultrasonic bath. The enzymatic dioxygenation was initiated by the addition of 2 mL of a lipoxygenase solution (*ca.* 2–3 mg/mL) to the stirred linolenic acid emulsion. Conversion was allowed to proceed for 20 min at approximately 0°C (ice bath) under continuous bubbling with oxygen. Finally, the reaction mixture was brought to a volume of 100 mL. This procedure resulted in a nearly complete conversion (>95%). The concentration of 13-HPOT was determined spectrophotometrically by measuring the absorbance at 234 nm against a blank containing no linolenic acid. For the calculation a molar extinction coefficient of 25,000 was used (17). Isomeric purity was analyzed after solid-phase extraction (RP-18 cartridges, Macherey & Nagel, Düren, Germany) by normal-phase HPLC (18), which revealed that 13-HPOT was the predominant isomer (>95%). 13-HPOT was always freshly prepared and used without further purification in the subsequent incubation experiments.

Germination of mung bean seeds and plant preparation. Mung bean seeds were soaked in water for 1 h and then placed on moist cotton wool. The seeds were germinated for 12–16 d at room temperature under normal light conditions without additional illumination and irrigated daily. In a typical procedure, 5 g mung bean seedlings were homogenized in 50 mL potassium phosphate–citrate buffer (pH 6.5, 0.1 wt% Triton X-100) with an Ultraturrax (Janke & Kunkel, Staufen i. Br., Germany) for 90 s. The homogenate was centrifuged at 4°C and 6400 × *g* for 20 min and the clear supernatant was used as crude enzyme extract.

Incubation and product isolation. Metabolism of the hydroperoxide was started by the addition of 10 mL of the 13-HPOT solution (about 50 µmol 13-HPOT) to the crude enzyme extract. The slight increase of pH caused by the alkaline precursor solution was immediately corrected with 0.1 M HCl to the desired pH of 6.5. Sealed flasks were incubated for 1 h at room temperature in a circular agitator. A control sample was treated in the same manner except that 10 mL of 0.1 M K_2HPO_4 buffer pH 9.0, instead of the precursor solution, were used. After acidification of the reaction mixture with 0.1 M HCl to pH 4.0, the products were extracted three times with 70 mL pentane/diethyl ether (1:1, vol/vol). The combined organic phases were dried over anhydrous sodium sulfate and brought to 250 mL with the extraction solvent. Subsequently, two methods of concentration were applied, depending on the kind of metabolite to be quantified. To determine volatile compounds, an aliquot (50 mL) was subjected to distillation using a Vigreux column (water bath temperature 40°C), which excluded a loss of volatiles but was relatively time-consuming. After concentration to a final volume of 1–2 mL, an internal standard (methyl nonanoate) was added and the sample was analyzed by GC.

For the analysis of nonvolatile metabolites the remaining extract (200 mL) was concentrated under vacuum at 35°C. The residue obtained after evaporation of the solvent was redissolved in 5 mL of diethyl ether and methylated with diazomethane. Subsequently, the solution was brought to 10 mL, and after suitable dilution with *n*-hexane, subjected to HPLC analysis.

Analysis of volatile compounds by GC. Volatiles were analyzed with a Fisons gas chromatograph GC 8180 (Mainz, Germany) equipped with a cool on-column injector and flame ionization detector. Compounds were separated on a Sato Wax capillary column (30 m × 0.32 mm, 0.45 µm film thick-

ness) from Sato (Mönchengladbach, Germany) with a temperature program from 40°C (held for 2 min) to 220°C at 4°C/min. The temperature of the detector was 270°C. Hydrogen was used as carrier gas at an inlet pressure of 50 kPa.

Analysis of nonvolatile compounds by HPLC–diode array detector (DAD)/ELSD. Analysis was performed with an HPLC apparatus from Jasco (Groβ-Umstadt, Germany), which consisted of a pump PU-980, degasser DG-980-50, gradient unit LG-980-02, DAD MD-910, and an injection valve from Rheodyne (Cotati, CA) with a 20-μL sample loop. An ELSD Sedex 55 from Sedere (Alfortville, France) was connected in series with the DAD.

Methylated oxylipins were separated on a Lichrospher 100 DIOL column (250 × 4 mm, 5 μm) from Merck (Darmstadt, Germany) at a flow rate of 0.4 mL/min. This flow rate was chosen with respect to a transfer of the conditions to the HPLC particle beam–mass spectrometry (PB–MS) system, because the particle beam interface operates best at low flow rates. The compounds were eluted isocratically for 10 min with *n*-hexane/2-propanol (98:2, vol/vol), then a linear increase of the gradient to *n*-hexane/2-propanol (85:15, vol/vol) within the next 25 min was followed by a washing step with *n*-hexane/2-propanol (50:50, vol/vol) for 20 min to elute Triton X-100. Attention had to be paid to the proper composition of the eluent, because slight changes at the beginning [e.g., *n*-hexane/2-propanol (95:5, vol/vol)] caused coelution or even an inversion of the elution order of some peaks. Metabolites were detected with a DAD (210 and 235 nm) as well as with an ELSD. The ELSD operated with compressed air as nebulization gas (2.0 bar) and with an evaporation temperature of 40°C. The gain was set to 8. Because reference substances were not commercially available (or in the case of 12-oxo-PDA for an extremely high price only), the detector was calibrated with methyl ricinoleate (methyl 12-hydroxy-9*Z*-octadecaenoate) and *n*-heptyl jasmonate. *n*-Heptyl jasmonate was prepared from methyl jasmonate (Aldrich, Deisenhofen, Germany) by transesterification with *n*-heptanol and a catalytic amount of H_2SO_4. Preparative thin-layer chromatography on silica plates with pentane/dichloromethane (8:2, vol/vol), R_f = 0.48–0.52, gave pure product (>98%, by GC, ratio *trans/cis* isomer: 95:5).

HPLC PB–MS. Oxylipins were identified by PB–MS. A Hewlett-Packard mass spectrometer 5989A (Palo Alto, CA) equipped with a particle beam interface 59980B was coupled to the HPLC system already described using the same chromatographic conditions. The interface operated with helium as nebulization gas at an inlet pressure of 415 kPa. Temperatures of the desolvation chamber, ion source, and quadrupole were 60, 250, and 100°C, respectively. Electron impact (EI) and chemical ionization (CI) spectra were recorded within a scan range of *m/z* 88–450 at an ionization energy of 70 and 100 eV, respectively. CI was performed with methane as ionization gas. The double-bond configuration of ketol derivatives was not determined explicitly. It was assumed that the stereochemistry of 12,13-EOT hydrolysis products was the same as reported for other plant sources (8,19).

Micropreparative gas chromatography. Methyl 12-oxo-10*E*-dodecenoate was isolated from the methylated organic extract by means of micropreparative gas chromatography and used as reference substance to determine its retention time in the HPLC system. Identity was confirmed by GC–MS analysis.

A MCS Gerstel Series II instrument (Gerstel, Mülheim/Ruhr, Germany) with a Hewlett-Packard 7673 autosampler, a Gerstel KAS-3 cold injection system, a Gerstel PFS fraction collector, and a Gerstel MCS 1.15 control and data acquisition system were employed. An OV 1 capillary column (3 m × 0.53 mm, 2-μm film thickness, Leupold, Fahrenzhausen-Weng, Germany) was connected to a BC CW 20 M capillary column (25 m × 0.53 mm, 1-μm film thickness, Leupold). Conditions were as follows: KAS: 40°C for 20 s, then raised by 10°C/s up to 230°C, there held for 120 s. Oven: 60°C for 4 min, then raised by 4°C/min up to 188°C and, in a second step, by 5°C/min up to 230°C, there held for 15 min. PFS: Supply pipe and distributor 220°C, trap cooling: 0°C. Flame-ionization detector: 250°C. Gases: Carrier gas H_2 at 5.0 mL/min, auxiliary gas H_2 at 1.0 mL/min, counter gas H_2 at 10.0 mL/min. Transfer of methyl 12-oxo-10*E*-dodecenoate from the KAS to the first column was allowed from 20 to 65 s after injection; from the first column to the second column from 29 to 31 min; from the second column to the trap from 35.62 to 36.75 min. The total injection volume was 12 μL per run.

GC–MS analysis. Collected HPLC fractions (methyl esters) were analyzed with and without conversion into methyl oxime and trimethyl silylether derivatives (2). GC–MS analysis was carried out as described elsewhere (20) except that a BC SE-54 capillary column (25 m × 0.32 mm, 0.4-μm film thickness, Leupold) and a split injector (T: 250°C) were used. The oven temperature was programmed from 60 to 270°C at 4°C/min.

RESULTS AND DISCUSSION

GC analysis of oxylipins. During investigations of the metabolism of 13-HPOT by mung bean seedlings we tried to quantify the oxylipins as methyl ester derivatives by GC. In the case of the α-ketol these attempts were not successful. The peak of the ketol was split into a group of peaks, suggesting isomerization of the double bond or decomposition on the capillary column. Such a phenomenon was also observed with 12-oxo-13-hydroxy-9*Z*-octadecenoic acid, the α-ketol arising from 12,13-EOD, when its methyl ester and trimethyl silylether derivative were analyzed (21). The authors pointed out that double-bond isomerization may also be caused by reagent treatment: When the double bond was saturated by hydrogenation prior to trimethylsilylation a single GC peak was obtained. In addition, under our GC conditions 12-oxo-PDA (methyl ester) gave two peaks when a split injector was used, indicating possibly a rearrangement of the *cis*-orientated side chains into the more stable *trans* form.

HPLC-DAD/ELSD analysis of 13-HPOT-derived metabo-

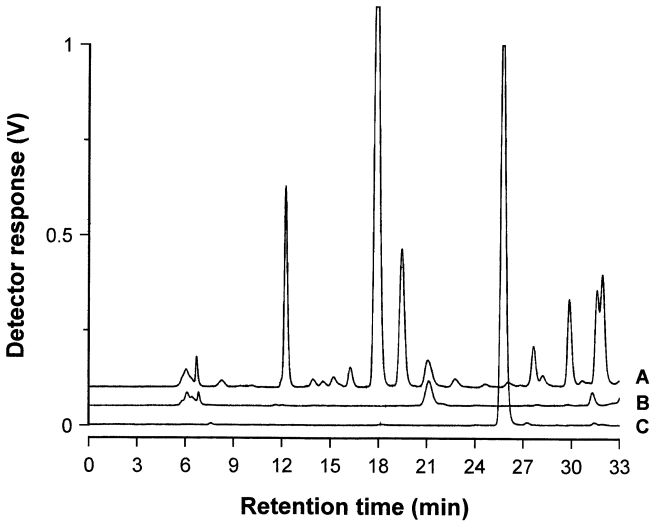


FIG. 1. Metabolism of 13*S*-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid (13-HPOT) by a crude enzyme extract from mung bean seedlings. Products were analyzed by normal-phase high-performance liquid chromatography (HPLC) using a Lichrospher DIOL column (Merck, Darmstadt, Germany) and an evaporative light-scattering detector (ELSD). Trace A: sample (crude enzyme extract plus 13-HPOT); Trace B: control sample (crude enzyme extract without 13-HPOT); Trace C: precursor 13-HPOT. For better presentation of the results an offset of detector response (about 0.1 and 0.05 V) was made for chromatograms A and B.

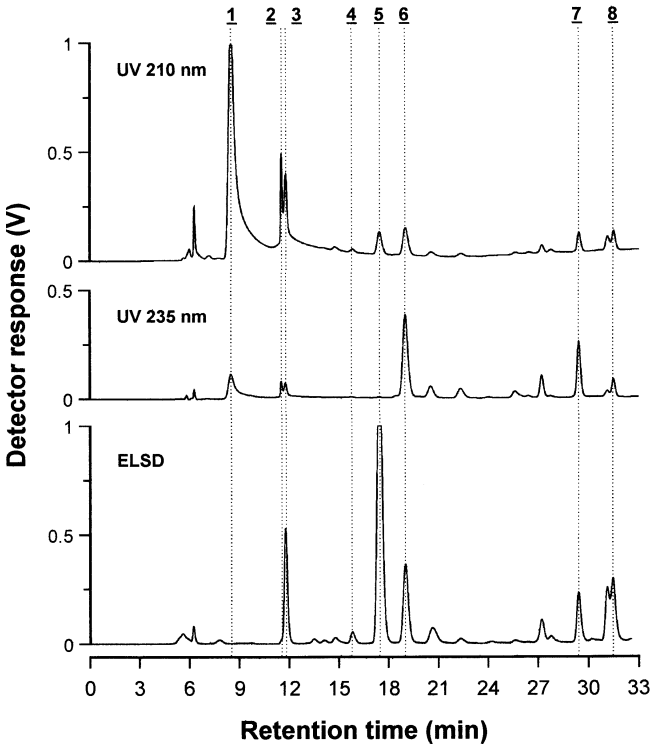


FIG. 2. Representative HPLC chromatograms of 13*S*-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid-derived oxylipins. Metabolites were detected with a diode array detector (DAD) at 210 and 235 nm and with an ELSD connected in series. For better comparability, the ELSD chromatogram, which had a detection time delay of about 0.4 min compared to the DAD, was shifted by this amount toward lower retention times. Peak numbers refer to the identified compounds as described in the text. UV: ultraviolet. See Figure 1 for abbreviations.

lites. A crude enzyme extract from mung bean seedlings was incubated with about 50 µmol 13-HPOT and, after extraction and subsequent methylation, subjected to HPLC analysis. In contrast to a control sample several additional peaks were detected, which could be successfully separated on a diol column (Fig. 1). The gradient elution system was able to resolve 13-HPOT from the products. Metabolites were monitored with a DAD and with an ELSD connected in series (Fig. 2). The wavelength of the DAD was set at 210 nm for the detection of metabolites without a pronounced ultraviolet chromophore and at 235 nm, which represents the absorption maximum of the precursor 13-HPOT (conjugated diene chromophore).

Both detectors differed significantly in their sensitivity for the various compounds (Fig. 2). Compounds **1** and **2**, the predominant peaks in the chromatogram acquired at 210 nm, were not detectable with the ELSD. Conversely, the detector response of the ELSD for compound **5** was about 15 times higher, based on peak area calculations, than the DAD at 210 nm. Such observations are not surprising when one considers the operation principle of an ELSD (22,23). The column effluent is nebulized to form a homogeneous mist of droplets. Subsequently, the aerosol enters a heated tube, where the solvent evaporates leaving a cloud of nonvolatile analyte particles. These particles pass through a light beam, and the scattered light, being proportional to the mass of the analytes, is detected by a photodiode. It follows that analytes, whose differences in volatility compared to the HPLC solvent are too small, cannot be detected with an ELSD or with drastically reduced sensitivity only. Obviously, **1** and **2** were volatile metabolites.

Identification of metabolites. To identify the products, the HPLC system was coupled without any alterations of the chromatographic conditions to a particle beam–mass spectrometer. Principles of LC–PB–MS have been reviewed in detail (24). As the sample inlet system of PB–MS is comparable to the ELSD just described, the ELSD served an important analytical pilot function for the PB–MS: Qualitatively identical chromatographic patterns were obtained with both detection systems. As a result, compounds which were detected with the ELSD were also present in the PB–MS chromatogram (not shown) and vice versa.

Because of their volatility, compounds **1** and **2** were not detectable by PB–MS and could not be identified by this way. Their structure was elucidated by GC–MS after collection of the HPLC effluent at about 9 and 12 min. Retention time and EI mass spectrum of **1** were identical to reference 2*E*-hexenal. GC–MS analysis of the second collected HPLC fraction (compounds **2** and **3**) revealed the presence of two peaks, which were identified as methyl 12-oxo-10*E*-dodecenoic acid (methyl 12-oxo-DDA) and methyl 12-oxo-phytodienoic acid (methyl 12-oxo-PDA). The EI mass spectrum of methyl 12-oxo-DDA

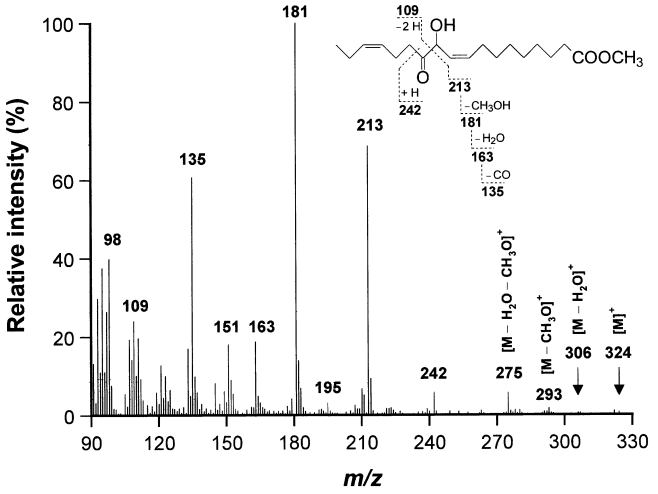


FIG. 3. HPLC particle beam mass spectrum (electron impact) and proposed fragmentation pattern of methyl 11-hydroxy-12-oxo-9*Z*,15*Z*-octadecadienoate. Ionization energy 70 eV. See Figure 1 for abbreviation.

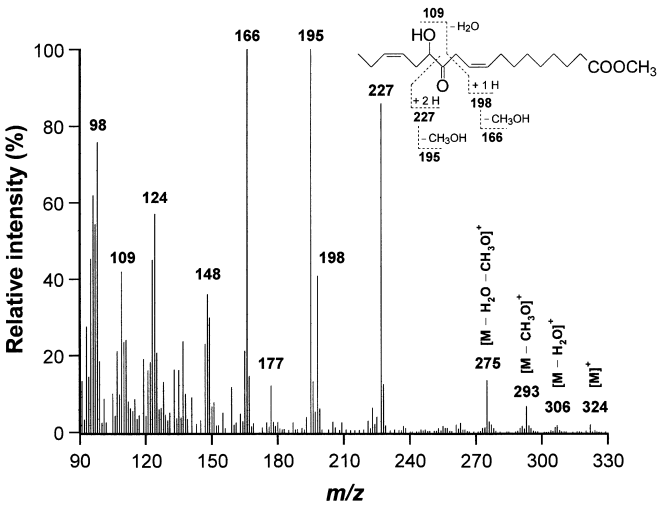


FIG. 4. HPLC particle beam mass spectrum (electron impact) and proposed fragmentation pattern of methyl 12-oxo-13-hydroxy-9*Z*,15*Z*-octadecadienoate. Ionization energy 70 eV. See Figure 1 for abbreviation.

was in agreement with that reported earlier (25). Methyl 12-oxo-PDA furnished an EI mass spectrum consistent with (26).

An exact assignment of these compounds to peak **2** or **3** in Figure 2 was difficult, because both occurred in the same HPLC fraction owing to their similar retention times. Therefore, methyl 12-oxo-DDA was isolated by micropreparative GC and used to determine its HPLC retention time. From this experiment and from cochromatography with the sample, the identity of peak **2** as methyl 12-oxo-DDA was demonstrated unambiguously.

EI and CI mass spectra of compounds **3**–**8** were recorded by PB–MS within a scan range of *m/z* 88–450 (exclusion of ions from the HPLC eluent). In addition, metabolites **4**–**8** were collected from the HPLC effluent and converted into trimethyl silylether derivatives with and without prior preparation of methyl oximes to obtain further proof of the position of keto and/or hydroxyl groups. GC–mass spectra of the derivatives were in agreement with the proposed structures (not all data shown). The CI mass spectrum [*m/z* (ion structure, percentage relative intensity)] of **3**, which was already identified by GC–MS as methyl 12-oxo-PDA, exhibited the expected quasi molecular ion *m/z* 307 ($[M + H]^+$, 100), methane adducts at *m/z* 335 ($[M + C_2H_5]^+$, 18), and 347 ($[M + C_3H_5]^+$, 6), and a peak at *m/z* 275 ($[M + H - CH_3OH]^+$, 17).

Figure 3 presents the EI mass spectrum of **4**. It showed clear similarities to the fragmentation pattern of 11-hydroxy-12-oxo-9*Z*,15*Z*-octadecadienoic acid (11,12-α-ketol) previously reported (8), indicating that **4** might be the methyl ester derivative. The CI mass spectrum consisted of ions at *m/z* 325 ($[M + H]^+$, 100), 307 ($[M + H - H_2O]^+$, 76), 293 ($[M + H - CH_3OH]^+$, 23), and 275 ($[M + H - H_2O - CH_3OH]^+$, 45) and supported the structure of **4** as methyl 11,12-α-ketol.

Metabolite **5** was identified as methyl 12,13-α-ketol. Its EI mass spectrum (Fig. 4) was in agreement with Reference 19. The molecular weight of **5** was confirmed by the CI mass spectrum (Fig. 5) showing an ion at *m/z* 325 ($[M + H]^+$, 19). Methyl 12,13-α-ketol underwent extensive elimination of water to form the most abundant ion at *m/z* 307 ($[M + H - H_2O]^+$, 100).

Compound **6** was characterized as methyl 13*S*-hydroxy-9*Z*,11*E*,15*Z*-octadecatrienoate (methyl 13-HOT). The EI mass spectrum presented peaks at *m/z* 308 ($[M]^+$, 1), 290 ($[M - H_2O]^+$, 7), 277 ($[M - CH_3O]^+$, 3), 239 ($[M - CH_3CH_2CH{=}CHCH_2]^+$, 66), 207 ($[M - CH_3CH_2CH{=}CHCH_2 - CH_3OH]^+$, 100), 189 ($[M - CH_3CH_2CH{=}CHCH_2 - CH_3OH - H_2O]^+$, 20), 171 (35), 147 (44), 95 (41), and 93 (43). The CI mass spectrum of **6** possessed an ion only at *m/z* 291 ($[M - H_2O + H]^+$, 100), due to elimination of water. Methane adducts were at *m/z* 319 ($[M - H_2O + C_2H_5]^+$, 7) and 331 ([M

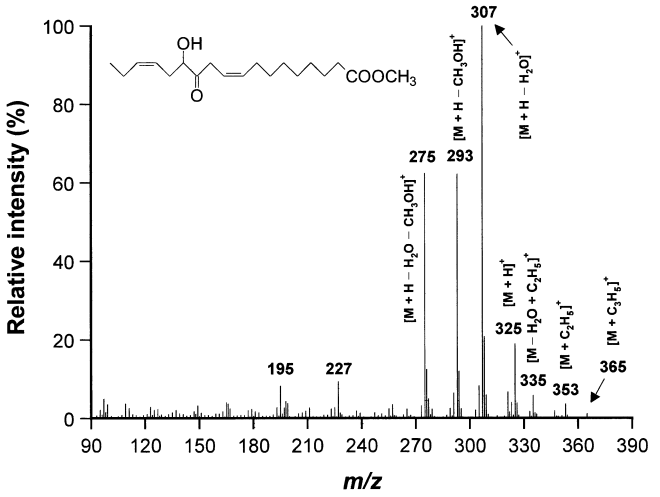


FIG. 5. HPLC particle beam mass spectrum (chemical ionization) and proposed fragmentation pattern of methyl 12-oxo-13-hydroxy-9*Z*,15*Z*-octadecadienoate. Chemical ionization was performed with methane gas as reactant gas. Ionization energy 100 eV. See Figure 1 for abbreviation.

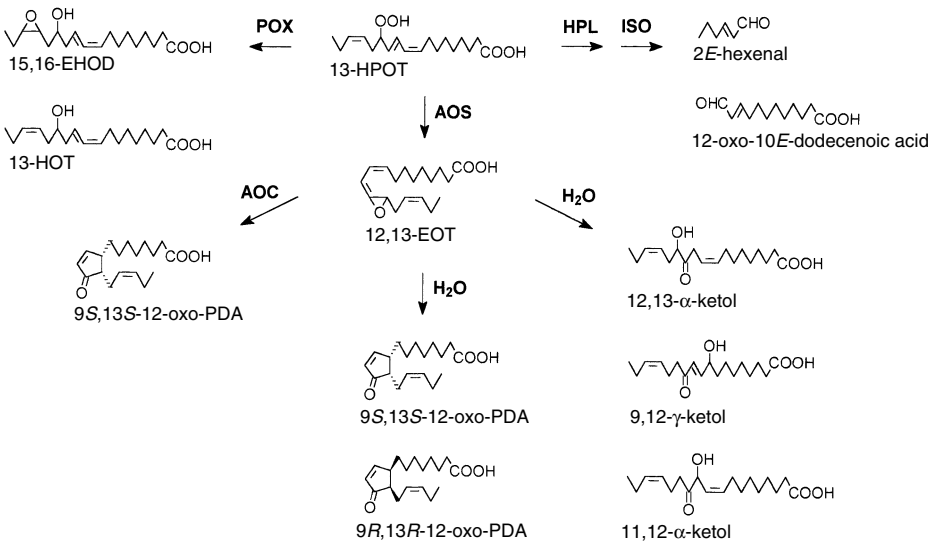
– $H_2O + C_3H_5]^+$, 2).

Metabolite **7** showed fragment ions at *m/z* 324 ($[M]^+$, 2), 306 ($[M - H_2O]^+$, 3), 275 ($[M - CH_3O - H_2O]^+$, 6), 239 ($[HC(OH)CH{=}CHCH{=}CH(CH_2)_7COOCH_3]^+$, 8), 237 (11), 207 ([*m/z* 239 – $CH_3OH]^+$, 27), 189 ([*m/z* 239 – CH_3OH – $H_2O]^+$, 7), 167 ($[M - (CH_2)_7COOCH_3]^+$, 20), 149 ([*m/z* 167 – $H_2O]^+$, 28), 135 (27), 128 (29), 121 (29), 109 (44), and 95 (100), suggesting a methyl 13-hydroxy-9,11-octadecadienoate with an additional oxygen atom. The CI mass spectrum had ions at *m/z* 325 ($[M + H]^+$, 5), 307 ($[M - H_2O + H]^+$, 65), 291 (48), 289 (45), 239 (100), 207 (37). A reliable identification was difficult until **7** was collected from the HPLC effluent and subsequently its trimethyl silylether derivative was prepared. The GC-mass spectrum (EI) obtained was qualitatively consistent with Reference 27. However, the ion intensities differed, because the spectrum published was recorded at an ionization energy of 16 eV only. From these findings **7** most likely represents methyl 13-hydroxy-15,16-epoxy-9*Z*,11*E*-octadecadienoic acid (methyl 15,16-EHOD).

The EI mass spectrum of **8** was similar at higher *m/z* values to the methylated ketols described before, indicating that **8** might also be a methylated ketol. Fragment ions of **8** were at *m/z* 324 (M^+, 2), 306 ($[M - H_2O]^+$, 3), 293 ($[M - CH_3O]^+$,7), 292 ($[M - CH_3OH]^+$, 6), 275 ($[M - CH_3O - H_2O]^+$, 10), 223 (9), 209 (14), 187 ($[HC(OH)(CH_2)_7COOCH_3]^+$, 29), 155 ([*m/z* 187 – $CH_3OH]^+$, 28), 137 ([*m/z* 187 – $CH_3OH - H_2O]^+$, 75), 111 ($[CH_3CH_2CH{=}CH(CH_2)_2CO]^+$, 48), and 95 (100). As mentioned in the introduction, the hydrolysis of the allene oxide 12,13-EOT is regularly accompanied by the formation of a 9,12-γ-ketol. Indeed, diagnostic ions at *m/z* 187 and 137 indicated that a hydroxyl group might be located at C_9. The position of the keto group was tentatively located at C_{12} owing to the ion at *m/z* 111. In CI mass spectrometry the quasi molecular ion of **8** was at *m/z* 325 ($[M + H]^+$, 100). Elimination of water and methanol resulted in peaks at *m/z* 307 ($[M + H - H_2O]^+$, 27), 293 ($[M + H - CH_3OH]^+$, 24), and 275 ($[M + H - H_2O - CH_3OH]^+$, 11). The GC–mass spectrum (EI) of trimethylsilylated **8** exhibited diagnostic ions at *m/z* 259 (hydroxyl group at C_9) and 285 (keto group at C_{12}), supporting the assumption that **8** was methyl 9,12-γ-ketol. Trimethylsilylated methyloxime derivative of **8** was consistent with Reference 28.

In summary, all of these products indicate that mainly three enzymes are involved in the metabolism of 13-HPOT in mung beans, namely, a hydroperoxide lyase, an allene oxide synthase, and a peroxygenase (Scheme 1: In this scheme, POX = peroxygenase; HPL = hydroperoxide lyase; ISO = isomerase; AOS = allene oxide synthase; and AOC = allene oxide cyclase.). 2*E*-Hexenal and 12-oxo-DDA originate from the action of a hydroperoxide lyase, a widely distributed enzyme in plants, on 13-HPOT (10–12). Depending on substrate specificity, hydroperoxide lyases cleave either C_9 or C_{13} hydroperoxides of polyunsaturated fatty acids, which contain a 1*Z*,4*Z*-pentadiene moiety, into C_9/C_6 aldehydes and C_9/C_{12} ω-oxo-acids. By using an extract from mung bean seedlings as an enzyme source, the initial cleavage products 3*Z*-hexenal and 12-oxo-9*Z*-dodecenoic acid were rapidly isomerized into the more stable *trans* isomers (unpublished data).

On account of the generation of 12,13-α-ketol and 9,12-γ-ketol, it is obvious that mung beans contain an allene oxide synthase, whose unstable product (12,13-EOT) undergoes immediate hydrolysis. A pathway for the formation of 11,12-α-ketol, which represents a novel hydrolysis product of 12,13-EOT, and of the other ketols has been proposed (8). 12-Oxo-PDA originates from the cyclization of the allene oxide. Since the enantiomeric composition of 12-oxo-PDA was not determined, it is unknown whether its cyclization occurs enzymatically, catalyzed by an allene oxide cyclase (4), or spon-



SCHEME 1

TABLE 1
Metabolism of 13-Hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic Acid (13-HPOT) by Mung Bean Seedlings[a]

Peak Number[b]	Metabolite	Amount (μmol)	Conversion rate (%)
1	2*E*-Hexenal	27.25	48.8
2	12-Oxo-10*E*-dodecenoic acid	n.d.	n.d.
3	12-Oxo-phytodienoic acid	2.05	3.7
4	11-Hydroxy-12-oxo-9*Z*,15*Z*-octadecadienoic acid	< 0.40	< 0.7
5	12-Oxo-13-hydroxy-9*Z*,15*Z*-octadecadienoic acid	3.36	6.0
6	13-Hydroxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid	1.22	2.2
7	15,16-Epoxy-13-hydroxy-9*Z*,11*E*-octadecadienoic acid	0.77	1.4
8	9-Hydroxy-12-oxo-10*E*,15*Z*-octadecadienoic acid	0.90	1.6

[a]A crude enzyme extract from mung bean seedlings was incubated with 55.8 μmol 13-HPOT at pH 6.5. Products were extracted with pentane/diethyl ether, methylated, and quantified by high-performance liquid chromatography–evaporative light-scattering detection as described in the Materials and Methods Section. 2*E*-Hexenal was determined by gas chromatographic analysis.
[b]Number of methylated metabolite in Figure 2. n.d.: not determined

taneously leading to the racemate.

A reduction of fatty acid hydroperoxides (13-HPOT, 13-HPOD) to the corresponding alcohols (13-HOT, 13-HOD) can be mediated by a peroxygenase (27) or a hydroperoxide-dependent epoxygenase (29). The terminal oxygen of the hydroperoxide group is transferred by an intermolecular and/or intramolecular mechanism to a substrate (e.g., unsaturated fatty acids, 13-HOT), which serves as oxygen acceptor, yielding hydroxy and concomitantly epoxy or epoxyhydroxy fatty acids (27,29). Indeed, the detection of 15,16-EHOD strongly implies the presence of a peroxygenase in mung beans. In a seed extract of soybean, a formation of 15,16-EHOD has also been reported (30). However, it cannot be excluded that 13-HOT is additionally generated by a nonenzymatic way. Incubation experiments performed in a phosphate–citrate buffer containing 10 mM ascorbic acid (to inhibit polyphenol oxidase) led to a 13-HOT concentration which was 2.8 times higher compared to a buffer without ascorbic acid, whereas the content of 15,16-EHOD was not affected (Rehbock, B., Gansser, D., and Berger, R.G., unpublished data). This suggests that reductants also are able to convert hydroperoxides into the corresponding alcohols.

Quantification of 13-HPOT-derived metabolites. Oxylipins were quantified with the ELSD based on the assumption that structurally similar compounds with nearly identical molecular weight caused the same detector response. Owing to a lack of reference substances, the detector was calibrated with methyl ricinoleate (methyl 12-hydroxy-9*Z*-octadecaenoate) and *n*-heptyl jasmonate. Methyl ricinoleate possesses most of the structural features of the methylated noncyclic oxylipins such as a hydroxyl group, double bond, and equivalent carbon-chain length. Similarly, the content of the cyclopentenoic fatty acid 12-oxo-PDA was calculated according to a calibration curve obtained with *n*-heptyl jasmonate (ratio *trans*/*cis* isomer: 95:5) to consider its cyclic structure. The GC–mass spectrum (EI) of the *trans* isomer [*m/z* (percentage relative intensity)] showed prominent ions at 308 ($[M]^+$, 41%), 290 (9), 261 (14), 240 (8), 210 (6), 193 (8), 192 (7), 191 (6), 164 (10), 163 (11), 151 (100), 150 (27), 83 (50), 57 (38), 55 (43), 43 (38), 41 (66), and of the *cis* isomer at *m/z* 308 ($[M]^+$, 41%), 290 (27), 261 (36), 240 (10), 210 (5), 193 (10), 192 (15), 191 (16), 164 (12), 163 (16), 151 (86), 95 (44), 83 (75), 57 (62), 55 (62), 43 (57), and 41 (100).

Both calibration curves exhibited a small range of linearity; beyond this range the curves became more and more sigmoidal. In the case of methyl ricinoleate, the detector response was linear from 10 to 70 μg/mL (injection volume: 20 μL). The linear regression equation (n = 10 data points), concentration (X) vs. peak area (Y), followed $Y = 4.19 \times 10^5 \times X - 3.78 \times 10^6$ (r = 0.999). In comparison to a calibration curve with methyl 13-HPOT, a nearly identical slope and intercept were achieved (data not shown), supporting the assumption that structurally related compounds possess the same detector response.

The slope and intercept of the curve for *n*-heptyl jasmonate varied markedly from that just described. The linear regression equation (n = 8 data points) was $Y = 2.73 \times 10^5 \times X - 5.41 \times 10^6$ (r = 0.995). Linearity of detector response comprised a range of 20–90 μg/mL (injection volume: 20 μL). Detection limits for *n*-heptyl jasmonate and methyl ricinoleate were at about 100 and 50 ng on column.

Based on these calibration curves the content of oxylipins, which were generated during the incubation of 13-HPOT with a crude enzyme extract from mung bean seedlings, was calculated. Additionally, 2*E*-hexenal was determined by gas chromatographic analysis. Results are summarized in Table 1. The high conversion rate of 13-HPOT into 2*E*-hexenal (48.8%) indicated that mung beans contain a considerable hydroperoxide lyase activity (Rehbock, B., Gansser, D., and Berger, R.G., manuscript in preparation), whereas the amount of oxylipins was relatively low (sum 14.9%). Although 13-HPOT was completely converted (Fig. 1, Trace A, no peak at 25.8 min), there remained a difference of about 36% in the balance. A probable explanation is the formation of one or several other products detectable by neither GC nor HPLC analysis. A control experiment in the absence of plant material revealed that a milky, solvent-insoluble precipitate was formed upon incubation and subsequent extraction of the precursor 13-HPOT. GC and HPLC chromatograms contained only traces of nonidentified products.

In conclusion, the HPLC system described in this study al-

lowed quantification and identification of methylated oxylipins and of the precursor 13-HPOT without additional derivatization. The application of an ELSD for HPLC analysis of oxylipins represented a significant improvement in sensitivity compared to an ultraviolet detector, especially in the case of the 12,13-α-ketol. In combination with the determination of volatile compounds by GC, this method proved to be an efficient tool for investigating the broad product spectrum of fatty acid hydroperoxide metabolism.

ACKNOWLEDGMENTS

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Use of an Improved Internal-Standard Method in the Quantitative Sterol Analyses of Phytoplankton and Oysters

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ABSTRACT: Most work reporting the sterol composition of living organisms has not been done quantitatively, although good quantitative data are available for fatty acids and many other cellular components using an internal-standard method that compensates for errors during gas chromatographic analysis. In this paper, we report on the use of 7-stigmastenyl acetate as an internal standard for sterol analysis in two species of phytoplankton and oysters produced with two different diets. This internal-standard method provides an internal standard for this entire process of analysis, not just the gas chromatographic analysis. When analyzing 50-μg samples of cholesterol acetate after hydrolysis and acetylation, about 30% of the sample was lost, resulting in a 30% error using the older external-standard method. Using the internal-standard method, the analysis error was less than 2%. Losses of sterol during analysis apparently are greater with plant and animal samples than with pure sterol standards. This internal-standard method was shown to be extremely useful, especially for samples with less than 500 μg of sterol. Finally, the standard error in sterol analysis is much lower when the internal-standard method is used, allowing statistical distinctions that are not possible otherwise. Use of 7-stigmastenyl acetate as an internal standard offers several advantages over the use of cholestane.

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Sterols are essential compounds for most living organisms, and their main role is as architectural components of membranes, as precursors of hormones, vitamins and bile acids, etc. (1). It is important therefore to quantitate sterols accurately from either plant, microbial, or animal tissues. In the past, total sterols were reported mainly on a weight basis, but after the advent of analysis by gas chromatography (GC), individual sterols were reported mainly as a percentage of total sterol on the recorded chromatogram.

The quantitative relationship between various sterols is important, but data on the quantity of total sterols on a weight or per-cell basis is lacking in most publications. Even when these data are calculated using an external quantitative standard, frequently a large error results because loss of sample during the analytical procedure is not accounted for. With the advent of capillary GC many analyses are performed with sample sizes in the milligram or even microgram ranges. In our laboratory, such analyses sometimes give highly variable quantitative results, with variability increasing with decreasing sample size. The use of cholestane as an internal gas chromatographic standard compensates for some of the error inherent in gas chromatographic analysis, but many potentially greater errors remain unaddressed (from saponification, extraction, purification and derivatization procedures prior to GC).

We have discovered that the quantity of sterols in phytoplankton species greatly affects their effectiveness as oyster food (2). The types and magnitude of variability in analyses of sterols from small samples noted above were not acceptable. Fortunately, this problem was addressed and solved some years ago in fatty acid analysis as follows: A known quantity of a fatty acid known to be absent in the sample is added to the sample as an internal standard prior to extraction and isolation of fatty acids. The amounts of other fatty acids present in the sample are calculated relative to the known quantity of the internal standard. Fatty acids have different response factors on GC depending upon chain length and unsaturation, and this is also taken into account (3). The internal-standard method also has been used for many years in medical analyses for cholesterol, especially for plasma cholesterol analysis (4–6), but it has not been adapted for sterol analysis of plant or animal tissues. The accuracy of sterol analyses from small samples would appear to be improved greatly by using an internal-standard system for the total analysis, not just the gas chromatographic analysis. This paper is an evaluation of an internal-standard method for sterol analyses in small samples.

MATERIALS AND METHODS

The purpose of our internal standard is to sustain losses proportionate to those of the sample during processing and analysis so that mathematical compensation can be made for the losses. The structure of the internal standard therefore should

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Abbreviations: GC, gas chromatography; T-ISO, *Isochrysis*; TTM, *Tetraselmis maculata*.

be as near as possible to the sterols being analyzed and yet not interfere with the final gas chromatographic analysis.

Selection and preparation of the internal standard. In our work with phytoplankton and oysters, a great variety of sterols is encountered. In red algae, for instance, many species have only C-27 Δ^5-sterols while green algae may have 27-, 28-, or 29-C sterols with various double-bond systems (7). Although a different standard must be used with some algae, most of them can be analyzed with the acetate of 7-stigmastenol (24-ethyl-5α-cholesten-7-3β-ol) as the internal standard, because it elutes after most sterol acetates on GC. (We use acetate derivatives since they chromatograph better on SPB-1 than free sterols.)

Unfortunately, 7-stigmastenyl acetate is not commercially available; it was obtained for this work by isolation of spinasterol from squash (8) seed. 7-Stigmastenyl acetate was prepared by hydrogenation of the acetate of spinasterol (24-ethyl-5α-cholesta-7,22-dien-3β-ol) over Raney nickel (Aldrich, Milwaukee, WI). Pure spinasteryl acetate (50 mg) in 25 mL ethyl acetate and 1 g Raney nickel (Aldrich) were hydrogenated for 24 h (1 atm, 25°C). Removal of catalyst and evaporation of the solvent yielded a mixture of steryl acetates which were purified by column chromatography over Anasil B (Analabs, Hamden, CT), eluting with 1% ether in hexane followed by recrystallization from methanol. This steryl acetate mixture contained 7-stigmastenyl acetate (93%) and 8(14)-stigmastenyl acetate (7%). It was reported previously that spinasteryl acetate and 7-dehydrostigmasteryl acetate, when hydrogenated by Raney nickel, always gave 8(14)-sterol as the principal product but that the addition of a little triethylamine prevented the formation of 8(14)-sterol (9,10). In these experiments, more than 90% of the hydrogenation product of spinasteryl acetate was 7-stigmastenyl acetate, with or without the addition of triethylamine; with the addition of triethylamine, a small amount of 8(14),22 always formed. In the course of hydrogenation, the 8(14),22 compound was converted to the 8(14) sterol. Pure 7-stigmastenyl acetate was obtained by further column chromatography on Anasil B.

Accuracy of analyses using internal and external standards and effect of sample size. From freshly prepared solutions of cholesteryl acetate and 7-stigmastenyl acetate, 10 samples were prepared to contain 50 µg of each sterol acetate. The solvent was evaporated and the samples were saponified for 1½ h with 1 M KOH in 80% aqueous ethanol at 65°C. Solvent was evaporated to reduce volume by one-half, and a few milliliters of H_2O were added. Sterols were extracted three times into *n*-hexane in a separatory funnel, fractions were combined, evaporated to dryness, dissolved in *n*-hexane, and chromatographed on a Sep-Pak (Waters Associates, Milford, MA) silicic acid cartridge with 2-mL elutions by *n*-hexane, chloroform, diethyl ether, and methanol. Sterols were eluted by diethyl ether and chromatographed as acetates [prepared by dissolving in pyridine/acetic anhydride (1:1) at room temperature overnight] on a Varian 3500 capillary gas chromatograph with an SPI injector and a Varian 4400 integrator (Palo Alto, CA). The injector received the sample at 95°C and was programmed to 300°C at 180° per min. The column was a 0.25 mm × 30 m fused-silica column with a 0.25-µm film of SPB-1. During chromatography, the column was programmed from 150–260°C at 10°C/min and held for 30 min. This experiment was repeated with samples of other quantities to determine how sample size affects percentage recovery.

Sterol analysis in Phaeodactylum *and* Tetraselmis *using the 7-stigmastenyl acetate internal standard.* Phytoplankton samples were from bacteria-free carboy cultures harvested in an N-deficient stationary phase. Freshly prepared 7-stigmastenyl acetate (250 µg) was added to a lyophilized sample of *Phaeodactylum tricornutum* (strain Phaeo) which was then extracted overnight in $CHCl_3/CH_3OH$ (2:1, vol/vol) using a Soxhlet extractor. The extract was divided into five aliquots, and the solvent was evaporated. Saponification, extraction of sterols, and acetylation of sterols and analysis on GC were as described previously except that extraction of sterols from the saponification mixture was better achieved with diethyl ether as compared to hexane.

Oyster analyses using the internal-standard method. Oysters analyzed had been fed a unialgal diet of either *Tetraselmis maculata* (strain TTM) or *Isochrysis* sp. (strain T-ISO) for 12 wk in molluscan rearing chambers at the Milford Laboratory (National Marine Fisheries Service, Milford, CT) (11). The internal standard of 150 µg of 7-stigmastenyl acetate was added to homogenized, fresh oyster tissue and refluxed in $CHCl_3/CH_3OH$ (2:1, vol/vol) for 8 h followed by filtration to remove the tissue (removal of tissue at this point improves the recovery of sterols). Saponification or other treatment of the tissue did not improve recovery. The crude extracts were divided into three aliquots, and the solvent was evaporated for saponification and further sterol isolation as described above.

RESULTS AND DISCUSSION

Cholesteryl acetete (50 µg) was saponified in the presence of the 7-stigmastenyl acetate internal standard, recovered, and reacetylated. This experiment allowed us to determine the accuracy and precision of our analysis with 10 replicates using an external-standard method vs. the internal-standard method. The external-standard method calculates the amount of cholesteryl acetate in the recovered sample by comparing the area of the recovered cholesteryl acetate on GC to the area of a known quantity of cholesteryl acetate. The problem with this method is that it does not account for the sample lost in the saponification–extraction–derivatization procedure. In the internal-standard method, the amount of cholesteryl acetate is calculated by comparing the area of the cholesteryl acetate peak with the area of the internal-standard peak (a known quantity). This method presumes that the relative losses of cholesteryl acetate and the internal standard are the same. When 5α-cholestane is the internal standard (6), the validity of this presumption is questionable since the recovery rate of a hydrocarbon and a sterol from an alkaline aqueous alcohol mixture should not be expected to always be similar.

In the experiments described above, 50-μg samples of cholesteryl acetate were saponified in the presence of 50 μg of the internal standard; then the cholesterol and 7-stigmastenol were recovered and acetylated as described. Data obtained by the internal-standard method had a lower standard error than those obtained by the external-standard method. This is because variable recoveries cause data scatter in the external-standard method while variable recoveries do not affect quantitative values in the internal-standard method. Analysis by the external-standard method shows 35.21 ± 1.37 μg of cholesteryl acetate in our 50-μg sample, while the internal-standard method reports 50.87 ± 0.70 μg.

In this experiment, the value obtained by the internal-standard method (50.87 ± 0.70 μg) is in error from the true value (50 μg) by only 1.8% while the value obtained by the external-standard method (35.21 ± 1.37 μg) is in error by 29.6%. The internal-standard value is more accurate and more precise, with smaller standard error (±0.70 vs. ±1.37). Other sample sizes were used to determine the effect of sample size on recoveries of cholesteryl acetate after saponification. Cholesteryl acetate samples of 500, 250, 100, 50, 25, 10, and 5 μg were saponified, and cholesteryl acetate recoveries were determined. Recoveries in the above series were 91, 87, 76, 69, 50, and 26%, respectively. Clearly, in this range of sample size, percentage losses increase as the sample size decreases, meaning that the external-standard method becomes more and more inaccurate as the sample size decreases below 500 μg.

Lyophilized samples of *P. tricornutum* (Phaeo) and *T. chui* (PLY-429) were extracted, and five aliquots of *Phaeodactylum* and three aliquots of *Tetraselmis* were taken for analysis by the two methods described above. Using the internal-standard method, the sterol composition of *Phaeodactylum* was 283.2 fg/cell (SE 1.1, $n = 5$) compared to 125.2 fg/cell (SE 3.1, $n = 5$) by the external-standard method. In *Tetraselmis* the sterol composition was 192.2 fg/cell (SE 1.8, $n = 3$) by the internal-standard method compared to 83.3 fg/cell (SE 8.5, $n = 3$) by the external-standard method. The amount of sterol recovered from each sample analyzed (as determined by the external-standard method) was 50 μg for *Phaeodactylum* and 83 μg for *Tetraselmis*. In a sample size of 100 μg of cholesteryl acetate (previous section), sample recoveries of about 75% were recorded. The much lower values recorded here by the external-standard method (44.2% from *Phaeodactylum* and 43.8% from *Tetraselmis*) may indicate that sample recoveries from saponified biological samples may be reduced compared to those in pure steryl acetate saponifications because of interference of biological compounds in the efficient extraction of sterols from saponification mixtures. Accordingly, the errors encountered with analyses of biological samples using the external-standard method may be much greater than they were calculated to be using pure cholesteryl acetate.

Extraction of sterols from fresh animal tissue is handled differently from that of dry phytoplankton, but the internal-standard method can be used to advantage here also. Six small individual oysters (each less than 100 mg fresh weight) were analyzed by each of our methods. Three oysters had been grown on a diet of *Isochrysis* (T-ISO), while the other three had been grown on a diet of *T. maculata* (TTM) for 12 wk. These two phytoplankton have very different sterol compositions with T-ISO having brassicasterol as about 99% of its total sterol and cholesterol present in traces (12). By contrast, TTM has 24-methylcholesterol as its major sterol, with much smaller amounts of 24-methylenecholesterol and cholesterol (13). The wild oyster contains nearly 40 sterols (14) with cholesterol, brassicasterol, 24-methylenecholesterol, and several other sterols as major components. The sterols of T-ISO-fed and TTM-fed oysters strongly reflected the sterols of their diet in agreement with the work of Berenberg and Patterson (15). In the oyster samples, the amount of sterol actually recovered using the external-standard method was in the range of 35–75 μg. The external-standard method reports values of 55 and 58% of the internal-standard values for sterol recovery for TTM-fed oysters and T-ISO-fed oysters, respectively. Here again the external-standard method clearly is inferior to the internal-standard method. The discrepancy between the two methods is not so great in the oyster samples as in the phytoplankton, even though the sterol sample is smaller with the oyster. A large lipid fraction (dry weight basis) and potentially more interfering substances in the extraction process in phytoplankton could be an explanation.

It is interesting to note that, in using the internal-standard method, it is possible to say that the total sterol (fresh weight basis) concentration of TTM-fed oysters (1932 μg/g) is greater than that of T-ISO-fed oysters (1777 μg/g). Using the external-standard method with its larger standard error, there are no statistical differences in total sterol concentration in the two sets of oysters (Table 1).

These experiments using the external-standard method and the internal-standard method for estimating the amount of cholesteryl acetate recovered from a saponification mixture show that with a 50 μg sample size the accuracy obtained by the internal-standard method was within 1.8% of the true value, while the error obtained by the external-standard method was 29.6%. The large error in the external-standard method is largely caused by losses of sample during preparation and analysis while the internal-standard method allows us to compensate for those losses. These errors are less with larger and greater with smaller samples with both methods, but the internal-standard method is superior by a wide margain at all levels tested. The advantage of the internal-standard method also is apparent with biological samples where the apparent loss of sample is even greater (with comparable size samples) than with the pure steryl acetate. Finally, the internal-standard method, with its much lower standard error, allows us to make statistical distinctions in sterol compositions of various treatments that we could successfully not do otherwise.

It is important to emphasize that when a steryl acetate such as 7-stigmastenyl acetate is used as an internal standard, it can compensate for errors due to absorption by tissue or glassware, losses due to spillage or air oxidation, or losses encountered in saponification, extraction of sterol from the saponification mixture, or derivatization. Although the traditional in-

TABLE 1
Quantitative Sterol Analysis of Oysters *(Crassostrea virginica)*[a] Comparing the Internal- and External-Standard Methods

	Oyster size[b]	Internal method[c]	External method[c]
TTM-fed oysters			
#1	68	1984	1026
#2	33	1900	1102
#3	33	1910	1036
Mean	45	1932 ± 26a,a	1053 ± 24b,a
T-ISO-fed oysters			
#1	43	1733	1227
#2	40	1766	877
#3	40	1831	1211
Mean	41	1776 ± 29a,b	1105 ± 114b,a

[a]The principal sterols of *Tetraselmis maculata* (TTM)-fed oysters were cholesterol, 24-methylcholesterol, and 24-methylenecholesterol, and the principal sterols of *Isochrysis* (T-ISO)-fed oysters were cholesterol and brassicasterol. Trace sterols were not included in these analyses.
[b]mg fresh weight.
[c]μg/g fresh weight. First letter shows results of analysis of variance (ANOVA) comparison ($P = 0.05$) between internal- and external-standard methods. Second letter shows results of ANOVA comparison between oysters fed different algae, using either internal- or external-standard method. A significant difference ($P = 0.02$) in total sterol of oysters fed TTM, as compared to T-ISO, was revealed using the internal-standard method; whereas, there was no significant difference using the external standard.

ternal-standard method was designed to compensate for errors in gas chromatographic analysis, it is obvious that to receive all the benefits of an internal standard listed above, it should (i) be added at the beginning of the analysis, (ii) be structurally similar to the compound being analyzed, and (iii) not interfere with the gas chromatographic analysis.

Although this method appears to be particularly useful for quantitative analysis of sterols from photosynthetic tissue, it should be equally applicable to other biological samples.

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REVIEW

High-Resolution Nuclear Magnetic Resonance Spectroscopy —Applications to Fatty Acids and Triacylglycerols

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ABSTRACT: During the past two decades, nuclear magnetic resonance spectroscopy (NMR) has played an ever-increasing role in the structural determination of fatty acids, fatty acid derivatives and analogues, and in the analysis of the structures of triacylglycerols including the quantitative analysis of lipid mixtures. This article discusses some of the results obtained through the application of the NMR technique to lipid molecules and reviews the literature. To maintain brevity, this article does not cover the underlying theory of NMR spectroscopy as numerous books devoted to modern NMR spectroscopy have been published.

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Early studies (1–7) of high-resolution ^{1}H nuclear magnetic resonance (NMR) permitted specific structural, kinetic, or equilibrium studies of individual compounds in pure state or in mixtures to be carried out. This technique was first used to determine both the degree of unsaturation in triacylglycerols and the average molecular weight of natural fats (8). Analysis of the positional distribution of fatty acids in triacylglycerols by ^{1}H NMR spectroscopy was performed with the help of shift reagents such as Eu(fod)$_3$, tris-(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)-europium(III) and Pr(fod)$_3$, tris-(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)-praseodymium(III) (9–14). These lanthanide shift reagents were Lewis acids with paramagnetic properties, and their action as shift reagents depended entirely on their ability to bind with the molecules as ligands in order to produce large chemical shift changes. Wedmid and Litchfield (10) have identified 2-isovaleroyl and 1,3-isovaleroyl triacylglycerol structures by high-resolution ^{1}H NMR in their studies of isovaleroyl lipids in dolphin, porpoise, and toothed whale fats. Differentiation between unsaturated and isomeric triacylglycerols, such as glycerol 1,3-dipalmitate-2-oleate and glycerol 1,2-dipalmitate-3-oleate, was also feasible by this technique (11,12). Furthermore, positional distribution of linoleate and linolenate chain in triacylglycerols was determined (13). Although this technique could be exploited to reveal structural details of triacylglycerols, the application was limited by the broadening of signals when shift reagents were used and handicapped by the narrow proton magnetic resonance range.

Taking advantage of the development of digital computers, pulse Fourier transform techniques and the availability of superconducting magnets, ^{13}C NMR technique complemented ^{1}H NMR technique in many ways. The natural abundance of ^{13}C (1.1%) in nature was compensated by the low possibility of ^{13}C-^{13}C spin-spin coupling and by broadband heteronuclear decoupling (i.e., elimination of all ^{1}H-^{13}C couplings) which resulted in sharp singlets for all ^{13}C absorptions, permitting small chemical shift differences to be measured. With advanced electronic techniques, it was also possible to run a ^{13}C NMR spectrum under conditions that allow for a quantitative integral, as in the case of ^{1}H NMR. Gated decoupling was used to remove the nuclear overhauser enhancement, and longer pulse delays were used to allow ^{13}C nuclei with long spin-lattice relaxation time to relax completely. These techniques required long experimental time (i.e., >12 h) to obtain a spectrum with suitable signal-to-noise ratio. However, with the use of relaxation reagents, such as tris-(acetylacetonato)-chromium(III) [Cr(acac)$_3$], the total time for collecting a spectrum was shortened to 1–2 h. (Note: relaxation reagents are paramagnetic substances capable of greatly reducing the relaxation times of substrate nuclei without inducing noticeable shifts.) The quantitative applications of ^{13}C NMR spectroscopy on lipid molecules were reviewed by Shoolery (15) with which the iodine values of fats and oils were determined accurately. As a complement to ^{1}H NMR, ^{13}C NMR offered a means of determining the composition of mixtures of fatty acids and lipid molecules in much greater detail. Gunstone published several valuable reviews, which dealt specifically with the use of high-resolution ^{13}C NMR technique in the analysis of lipid mixtures (16–19).

High-resolution ^{1}H NMR spectroscopy has had limited use in fatty acid analysis owing to the small range of chemical shifts covered by protons, which resulted in the small number of signals in the proton spectrum. The splitting patterns of proton signals, however, can provide unique structural properties of lipid molecules under investigation (20). ^{13}C NMR

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Abbreviations: 2D COSY, two-dimensional correlation spectra; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HMBC, heteronuclear multiple bonds correlation; HSQC, heteronuclear single quantum correlation; INADEQUATE, incredible natural abundance double quantum transfer experiment; NMR, nuclear magnetic resonance.

spectra of fatty acids provided a large number of signals spread over a wide range of chemical shifts, which made the spectrum appear complicated but much more informative. Techniques of correlating signals between ^{1}H and ^{13}C NMR spectra provided two-dimensional correlation spectra (2D COSY), which permitted confirmation of signals. Other techniques, such as INADEQUATE (incredible natural abundance double quantum transfer experiment), HSQC (heteronuclear single quantum correlation), HMQC (heteronuclear multiple quantum correlation) and HMBC (heteronuclear multiple bonds correlation), are some of the latest techniques in NMR spectroscopy from which structural details can be derived in great detail. Many of these NMR experiments can be readily carried out with the latest models of high-field NMR instruments, and such operations have become more and more common in the past 5 yr.

STUDIES OF INDIVIDUAL UNSATURATED FATTY ACIDS AND ESTERS

Olefinic fatty acids. Many long-chain unsaturated fatty acids are claimed to have been "identified" by gas–liquid chromatography. However, in such analyses, it is often presumed that the unsaturated centers of the fatty acids are located at the common position(s) of the alkyl chain, such as the Δ^{9} (oleic acid) and $\Delta^{9,12}$ (linoleic acid) positions. Fatty acids with double bonds in other positions cannot be readily determined from their gas chromatography retention times (21). Application of other analytical techniques, such as chemical oxidation, infrared and ultraviolet spectroscopy and mass spectrometry, are therefore necessary to establish the positions and the configurations of the unsaturated centers in the alkyl chain (22–25).

The high resolving power of ^{13}C NMR spectroscopy allows many of the positional isomers of long-chain fatty acids or esters to be differentiated. For example, in the spectrum of methyl oleate (Fig. 1), the shifts of the olefinic carbon atoms appear at δ_C 129.76 and 130.01 for the C-9 and C-10 carbon atoms, respectively. The terminal methyl carbon shift (C-18) is found at δ_C 14.13, while the carbonyl carbon of the ester function (C-1) gives rise to a signal at δ_C 174.31. The methyl carbon of the ester appears at δ_C 51.46. Four methylene groups are readily identified: δ_C 34.12 (C-2), 24.98 (C-3), 31.94 (C-16), and 22.72 (C-17). The allylic carbon atoms adjacent to the *cis*-double bond are found at δ_C 27.26 and 27.19 (C-8 and C-11). The remaining methylene carbon shifts are more difficult to identify because they are clustered within a small region of the spectrum at about δ_C 29.12–29.80 (often referred to as the "methylene envelope"). In the case of methyl petroselinate, 18:1(6*c*) [where the first number denotes the chain length of the fatty acid, the number after the colon denotes the number of unsaturation, and the number within the parentheses indicates the position of the unsaturation, *c* for *cis*, *t* for *trans,* and *a* for acetylene (Fig. 2)], the

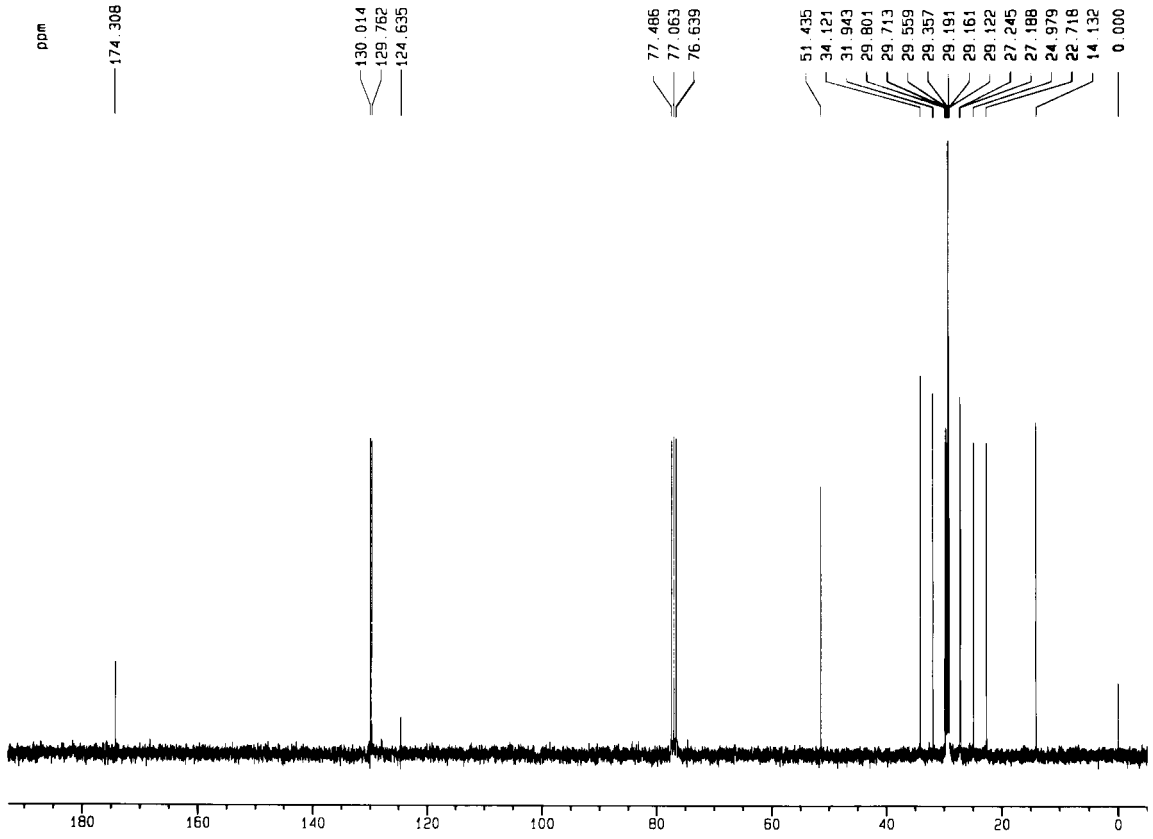


FIG. 1. ^{13}C nuclear magnetic resonance (NMR) spectrum of methyl oleate, 18:1(9*c*); *c, cis.*

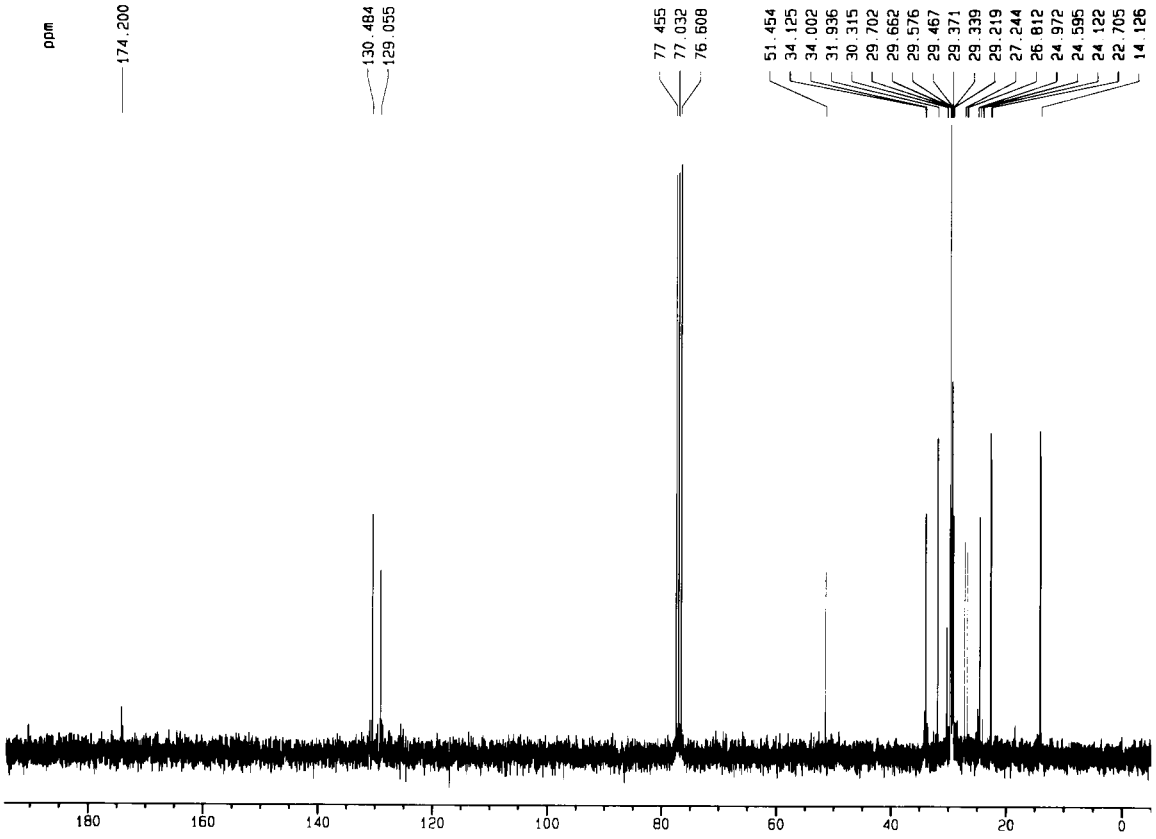


FIG. 2. ^{13}C NMR spectrum of methyl petroselinate, 18:1(6*c*). See Figure 1 for abbreviations.

shifts of the olefinic carbon atoms appear at δ_C 130.48 and 129.06 for the C-6 and C-7, respectively. The difference of these two chemical shift values appears quite constant and is characteristic of the said positional isomer, which allows such data to be used for the location of the position of the olefinic system in the alkyl chain (Δ^6 position in this case). The rest of the signals are similar to those of methyl oleate for the shifts of the corresponding carbon nuclei.

Bus *et al.* (26,27) studied hundreds of fatty acid methyl esters with *cis*- and *trans*-double bonds and triple bonds at various positions and in many different combinations in the alkyl chain. A comprehensive set of chemical shift parameters has been developed for the various substituents. With the aid of these parameters, the chemical shifts of all methyl, methylene, and carbonyl carbon nuclei can be predicted with a high degree of accuracy. A similar approach has been adopted by Gunstone *et al.* (28) in the study of the carbon chemical shifts of more than 90 *cis*- and *trans*-long-chain alkenoic acids. In the case of medium-chain fatty esters, such as the positional isomers of methyl *cis*-undecenoate, the shifts of the olefinic carbon atoms are all fully resolved, and each isomer can be identified by this technique. In the longer chain series, *viz.* methyl octadecenoate isomers (18:1), the olefinic carbon atoms can be marginally resolved as far away as the Δ^{11} position from the carboxylate end. One characteristic feature of the carbon shift data of these positional isomers is that the difference between the shift values of the two olefinic carbon nuclei diminishes gradually with distance of the olefinic system from the carboxylate end. By studying these minute differences, it is possible to define the position of the olefinic bond in the alkyl chain of the fatty acid or ester as illustrated in the case of 18:1(9*c*) and 18:1(6*c*), as discussed above. The effects of the end group on the shifts of the various carbon atoms of the alkyl chain have been interpreted in terms of steric and linear electric field effects by Batchelor *et al.* (29).

In order to predict the position of double bonds in an alkyl chain and to assign the numerous signals arising from the ^{13}C NMR analysis of an unsaturated fatty acid or ester, several authors have given values for the effect that the end groups and the double bond have on the chemical shifts of nearby carbon atoms. By applying the additivity rule, the shifts of each carbon atom of the methylene groups can be estimated by adding or subtracting the parameters, depending on the location of the carbon nucleus from the affecting group(s) (26, 27,30,31). Bus *et al.* (26,27) and Gunstone *et al.* (28) have also reported the carbon chemical shifts of the olefinic carbon atoms of a large number of polyunsaturated fatty esters. The carbon shifts of a few polyunsaturated fatty esters are presented in Table 1.

The assignments of the shifts of the carbon nuclei of such positional isomers of fatty acids or esters by ^{13}C NMR spectroscopy have been possible by the comparative study of complete series of positional isomers, many of which have been obtained by total synthesis. With reference to these car-

TABLE 1
^{13}C NMR Chemical Shift Values of Some Polyunsaturated Fatty Acid Esters (Refs. 26,27)[a]

Carbon nuclei	Compound		
	18:2(9*c*,12*c*)	18:2(5*t*,9*c*)	18:3(6*c*,9*c*,12*c*)
C-1	174.10	174.15	173.83
C-2	34.10	33.45	33.99
C-3	25.05	24.85	24.70
C-4	29.30	32.00	29.23
C-5	29.30	129.40	26.96
C-6	29.30	131.15	129.60
C-7	29.75	32.75	128.38
C-8	27.35	27.35	25.74
C-9	130.05	129.10	128.14
C-10	128.25	130.45	128.46
C-11	25.75	27.35	25.74
C-12	128.10	29.80	127.74
C-13	130.20	29.40	130.42
C-14	27.35	29.60	27.33
C-15	29.45	29.40	29.44
C-16	31.70	32.00	31.64
C-17	22.70	22.75	22.66
C-18	14.10	14.10	14.07
$COOCH_3$	51.30	51.40	51.31

[a]*c, cis*; *t, trans*; NMR, nuclear magnetic resonance.

TABLE 2
^{13}C NMR Chemical Shift Values of Conjugated Linoleic Acid Esters (Ref. 41)[a]

Carbon nucleus	Compound			
	18:2(9*c*,11*t*)	18:2(9*t*,11*c*)	18:2(9*t*,11*t*)	18:2(9*c*,11*c*)
C-1	174.32	174.34	174.22	174.27
C-2	34.10	34.10	34.09	34.10
C-3	24.95	24.95	24.98	24.97
C-4	29.06	28.97	29.04	29.14
C-5/C-7	29.12–29.67	29.13–29.77	29.14–29.77	29.11–29.60
C-8	27.66	32.86	32.61	27.46
C-9	129.89	134.51	132.16[a]	131.87[c]
C-10	128.71	125.72	130.37[b]	123.58[d]
C-11	125.58	128.57	130.51[b]	123.72[d]
C-12	134.76	130.17	132.43[a]	132.14[c]
C-13	32.92	27.72	32.68	27.54
C-14	29.41	29.73	29.40	29.68
C-15	28.95	28.97	28.97	29.04
C-16	31.77	31.77	31.82	31.81
C-17	22.65	22.65	22.68	22.69
C-18	14.12	14.12	14.13	14.13
$COOCH_3$	51.44	51.45	51.39	51.42

[a]See Table 1 for abbreviations. [a-a, b-b, c-c, d-d]Interchangeable.

bon chemical shift values, the structures of many polyunsaturated fatty acids have been elucidated or confirmed, *inter alia* the presence of 20:4(5*c*,11*c*,14*c*,17*c*) in the seed oil of *Biota orientalis* (32), and 18:4(6*c*,9*c*,12*c*,15*c*) in the seed oil of *Ribes nigrum* (33). The ^{13}C NMR properties of a number of geometric isomers of methylene-interrupted polyolefinic fatty acids have been reported. The spectral properties of 18:2(9*c*,12*t*) and 18:2(9*t*,12*c*) show unique carbon shifts for the two different geometries of the double bonds (34). In these isomers, the shifts of the methylene carbon atoms adjacent to the *cis*-double bond appear characteristically at about δ_C 27.1, while those adjacent to the *trans*-double bond are found at about δ_C 32.5. The shift of the carbon atom of the methylene between the two double bonds of 18:2(9*t*,12*c*) appears at δ_C 30.5.

The carbon shifts of the eight possible geometric isomers of 18:3(9,12,15) have been reported by Rakoff and Emken (35). The spectral properties of 20:3(5*c*,8*c*,14*c*) and 20:3(5*c*,11*c*,14*c*) (36), and 20:5(5*c*,8*c*,11*t*,14*c*,17*c*) and 20:5(5*c*,8*c*,11*t*,14*c*,17*t*) (37) have been studied. The ^{1}H and ^{13}C NMR spectra of six n-3 polyene esters (18:3, 18:4, 20:4, 20:5, 22:5, and 22:6) have been reported and discussed (38). The spectral properties of [1-^{14}C]linoleic acid and arachidonic acid, and some 18:3 isomers were also described (39,40).

We recently reported the synthesis and ^{13}C NMR properties of all four isomers of conjugated linoleic acid esters (41). The chemical shifts for these four isomers are given in Table 2. The assignments of the various signals were accomplished by using a combination of 2D COSY techniques (^{13}C-^{1}H COSY , INADEQUATE, and HMBC correlation experiments). To explain the detailed interpretation of the NMR spectra, the case of 18:2(9*c*,11*t*) was used as an example. The ^{1}H NMR spectrum of 18:2(9*c*,11*t*) showed four distinct signals for the four olefinic protons. These signals appeared as two multiplets (δ_H 5.32 and 5.65) and two triplets (δ_H 5.82, J = 5.4 Hz, 6.24, J = 12.9 Hz), which corresponded to the shifts of the protons of the "outer" (9-*H*, 12-*H*) and "inner" (10-*H*, 11-*H*) positioned olefinic protons of the conjugated diene system, respectively. The large J constant of the triplet at δ_H 6.24 was an indication that this signal arose most probably from one of the protons of the *trans*-double bond (i.e., 11-*H*). The ^{13}C NMR spectrum showed four signals in the olefinic region: δ_C 125.58, 128.71, 129.89, and 134.76. From the ^{13}C-^{1}H NMR COSY correlation spectrum (Fig. 3), it was clear that the triplet at δ_H 6.24 was correlated to the carbon at δ_C 125.58, and the triplet at δ_H 5.82 to the carbon at δ_C 128.71. These correlations therefore determined the shifts of the "inner" carbon atoms (C-10, C-11) at δ_C 128.71 and 125.58. The multiplets at δ_H 5.65 and 5.32 were correlated to the carbon atoms at 129.89 and 134.76, respectively, which determined the shifts of the "outer" positioned carbon atoms (C-9, C-12).

This spectrum also showed a correlation of the allylic carbon atoms (signals at δ_C 27.66 and 32.92) with the proton signals at δ_H 2.0 and 2.1 (multiplets). From the INADEQUATE spectrum (Fig. 4), which showed connections between one carbon nucleus with the adjacent carbon nucleus, the signal at δ_C 134.76 had correlations with two carbon atoms, *viz.* δ_C 125.58 and 32.92. The latter signal was characteristic of the shift of the allylic methylene carbon atom adjacent to a *trans*-double bond, which meant that the signal at δ_C 134.76 arose from the shift of the "outer" carbon of the *trans*-double bond (i.e., C-12). As the signal at δ_C 134.76 was also correlated to δ_C 125.58, this inferred that the latter signal was due to the shift of C-11 (one of the "inner" carbon atoms of the diene

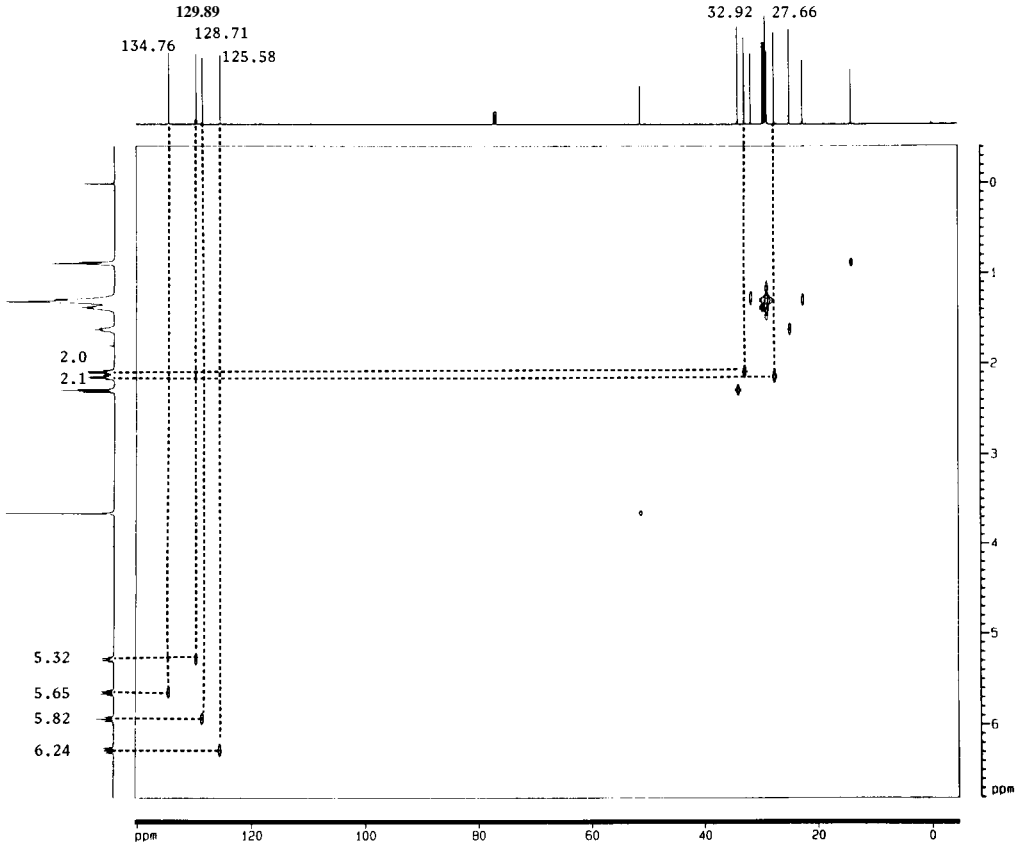


FIG. 3. ^{1}H-^{13}C NMR correlation (COSY) spectrum of 18:2(9*c*,11*t*); *t, trans*. See Figure 1 for other abbreviations.

system). The signal at δ_C 129.89 was correlated to that of δ_C 27.66, the latter being the signal arising from the allylic methylene carbon adjacent to the *cis*-double bond. Thus, the signal at δ_C 129.89 was due to the remaining "outer" signal of the diene system (i.e., C-9). The spectrum further showed a correlation between the signal at δ_C 125.58 (C-11) and that of δ_C 128.71. The latter signal was therefore due to the shift of the C-10 carbon nucleus. From the INADEQUATE spectrum, it was therefore possible to assign unambiguously the four carbon atoms of the *cis,trans*-diene system as: δ_C 129.89 (C-9), 128.71 (C-10), 125.58 (C-11), and 134.76 (C-12). Referring to the ^{13}C-^{1}H COSY spectrum, it is now possible to correlate the signals of the various olefinic carbon atoms with the corresponding proton signals. The proton shifts of the olefinic protons are therefore confirmed as: δ_H 5.32 (9-*H*), 5.65 (12-*H*), 5.82 (10-*H*), and 6.24 (11-*H*). In addition, it is also possible to assign the signals for the 8-*H* and 13-*H* protons as appearing at δ_H 2.1 and 2.0, respectively.

To reconfirm these assignments, the HMBC spectrum was recorded (Fig. 5). This technique shows correlations of nuclei (carbon to carbon or carbon to proton) which are two or three bonds apart. The signal at δ_C 125.58 (C-11) was correlated to the proton signals at δ_H 5.82 (*t*, 10-*H*), 5.32 (*m*, 9-*H*), and also to the signal at 2.0 (*m*, 13-*H*). The signal at δ_C 128.71 (C-10) was correlated to the proton at δ_H 5.65 (*m*, 12-*H*) and to the protons at δ_H 2.1 (*m*, 8-*H*). The signal at δ_C 129.89 (C-9) was correlated (weak signal) to the proton signal at δ_H 6.24 (*t*, 11-*H*). And the signal at δ_C 134.76 (C-12) was correlated to the proton signals at δ_H 5.82 (*t*, 10-*H*) and also to δ_H 2.0 (*m*, 13-*H*). Furthermore, the carbon signal at δ_C 32.92 (13-C) was correlated to the proton signals at δ_H 6.24 (*t*, 11-*H*) and 5.65 (*m*, 12-*H*), while the signal at δ_C 27.66 (C-8) was correlated to δ_H 5.82 (*t*, 10-*H*). These two- or three-bond connections reconfirmed the assignments of the carbon atom of the 9-*cis,*11-*trans*-diene system.

In the structural elucidation of the presence of α- and β-eleostearic acid [18:3(9*c*,11*t*,13*t*) and 18:3(9*t*,11*t*,13*t*), respectively] in the oil from oiticica, *Licania rigida* Benth (Chrysobalanaceae), Bergter and Seidl (42) used a combination of off-resonance decoupling and lanthanide-induced shifts coupled to polarization by a remote keto group to establish the correct order of the conjugated olefinic carbons by ^{13}C NMR spectroscopy. A C_{18} conjugated tetraenoic acid [18:4(8*c*,10*c*,12*c*,14*t*)] and crepenynic acid [18:2(9*c*,12*a*)] are the major fatty acid components in *Ixora chinensis* seed oil. From the ^{13}C NMR spectrum of the oil, the presence of crepenynic acid was confirmed by the signals at δ_C 78.29, 80.05, 125.28, and 131.40 for C-9, C-10, C-12, and C-13, respectively. The structure of the conjugated tetraene fatty acid was elucidated based on the signals for the olefinic carbon atoms (43).

Two new fatty acid metabolites, 20:5(5*c*,7*t*,9*t*,14*c*,17*c*) and

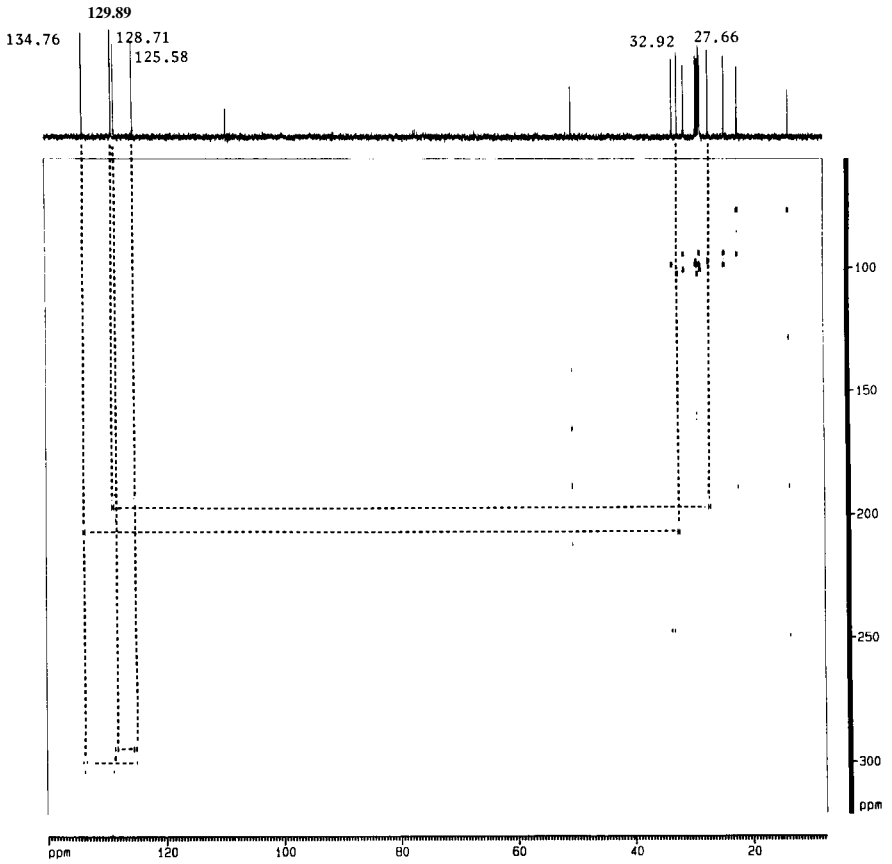


FIG. 4. Incredible natural abundance double quantum transfer experiment spectrum of 18:2(9*c*,11*t*). See Figures 1 and 3 for abbreviations.

20:5(5*t*,7*t*,9*t*,14*c*,17*c*), were isolated from the temperate red marine alga, *Ptilota filicina* (Ceramiales, Rhodophyta). The structures of these compounds were deduced from detailed ^{1}H, ^{13}C NMR, and two-dimensional NMR experiments (44). Aursand and Grasdalen (45) reported the carbon shift of the olefinic carbon atoms of n-3 polyene acids (22:6, 20:5, 18:4, 18:3), while Takagi *et al*. (46) described the shifts of a 24:6 polyunsaturated fatty ester and some 16:3 and 16:4 isomers in fish oils (47).

Acetylenic fatty acids. The ^{13}C NMR spectral analysis of acetylenic fatty acid esters was characterized by the signals at about δ_C 80 for the shifts of the carbon atoms of the acetylenic bond. Similar to the shifts of the olefinic carbon atoms, the effect of the carboxylate group also influenced the shift values of these nuclei, depending on the position of the acetylenic bond in the alkyl chain. The presence of an acetylenic bond in a fatty acid was further characterized by the shifts of the carbon atom of the methylene groups adjacent to the acetylenic bond, which appeared in the upfield region of the spectrum at about δ_C 18 (26,27,30).

The effects of the ester and terminal methyl group on the shifts of the acetylenic carbons allowed all isomers of methyl undecynoate to be differentiated from the shifts of the acetylenic carbon atoms (26). The position of the acetylenic bond in methyl octadecynoates was located as remote as the Δ^{10} position from the carboxylate group by examining the shift position of the acetylenic carbon atoms in the spectrum and from the difference of these shift values (30). The ^{13}C NMR spectral results of dimethylene-interrupted octadecadiynoates (26) and those of the conjugated diacetylenic fatty esters (48) also were reported.

The synthesis of acetylenic fatty acids (e.g., 2-pentadecynoic acid), which are potential inhibitors of the β-oxidation step in the biosynthesis of certain sex pheromones of the Egyptian armyworm *Spodoptera littoralis,* was reported. The position of the acetylenic bond in these fatty acids was readily identified by ^{13}C NMR spectroscopic analysis from the shifts of the acetylenic carbon atoms, which appeared at δ_C 72.5 (C-2) and 92.7 (C-3) for the Δ^2 acetylenic bond (49). The structure of a new polyunsaturated fatty acid, containing an acetylenic bond, was isolated from the thalli of *Riccia fluitans*. The structure of this acid, 18:2(6*a*,9*c*), was established by spectroscopic methods (50).

Deuterated long-chain fatty acids. Specifically deuterated long-chain fatty acids, either as free acids or chemically incorporated in phospholipids, frequently have been used in the investigation of lipid bilayers (membranes) by deuterium NMR (51–53). Many reports have been made on the synthesis of deuterated fatty acids with studies of the ^{13}C NMR properties of such analogs (54–56). Fatty acids which have

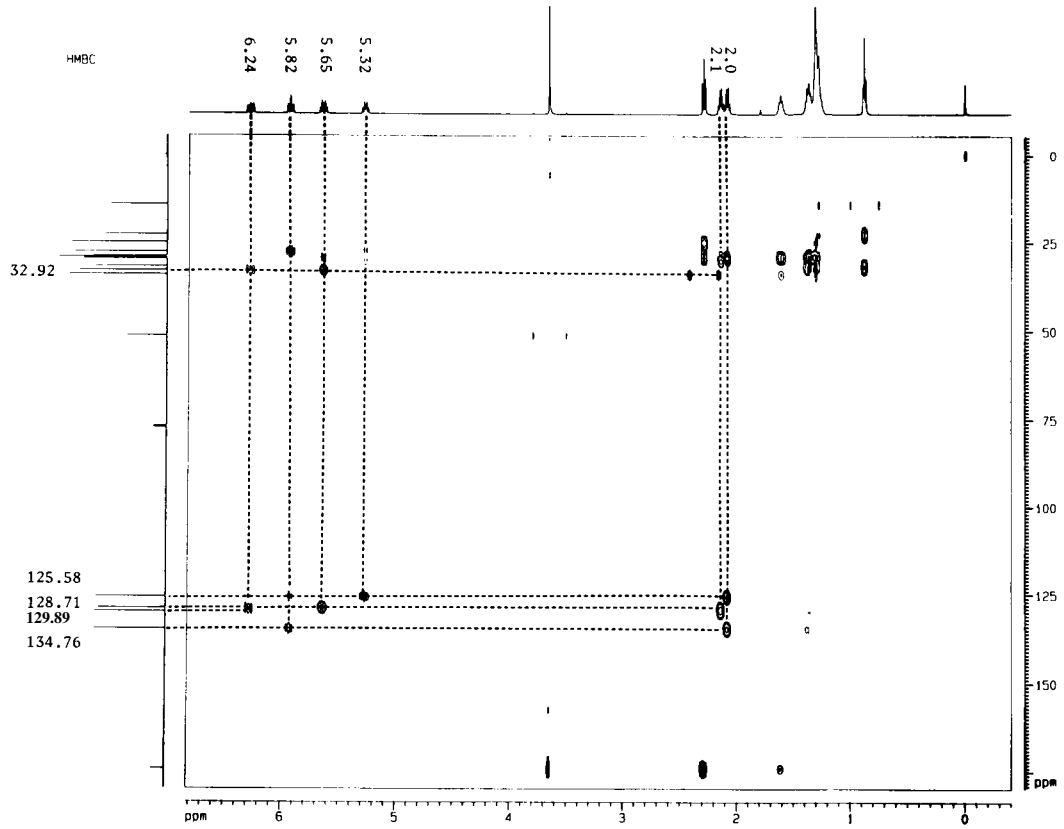


FIG. 5. Heteronuclear multiple bonds correlation spectrum of 18:2(9*c*,11*t*). See Figures 1 and 3 for abbreviations.

two deuterons on a specific carbon atom of the alkyl chain showed two different quadrupole splittings due to the nonequivalence of the deuterons (57,58). Consequently, the covalent attachment of two deuterons to a methylene carbon resulted in loss of signal intensity as a result of an increase in the relaxation time (T_1) and an increase in the line width of the resonance due to ^{13}C-^{2}H coupling (59,60). Signals from the deuterated carbon atoms were therefore absent or very broad, as illustrated in Figure 6 for the spectra of the nondeuterated and deuterated ethyl 8,11,14-eicosatrienoate-19,19,20,20-d_4 (61). The biosynthesis and characterization of a series of deuterated 18:2(6*c*,9*c*) by ^{13}C NMR spectroscopic analysis were carried out using deuterated fatty acids labeled at the C_4, C_5, C_6, C_7, C_8,C_{11}, C_{14}, or C_{18} positions (62). These acids were biosynthesized by supplementing cultures of the protozoan *Tetrahymena* with the corresponding deuterated 18:1(9*c*). The results illustrated the utility of a biological approach for the synthesis of deuterated polyunsaturated fatty acids in yields suitable for ^{2}H NMR studies.

An interesting feature of *gem*-dideuterated fatty acids or esters in the ^{13}C NMR spectral analysis was the deuterium isotope effects of the deuterons on the shift of the adjacent methylene carbon atoms. The average value for the second atom effect was −0.20 (upfield) and for the third atom the effect was −0.05 ppm. Thus the signals for the methylene carbon atoms adjacent to the deuterated methylene were readily identified by comparison to the nondeuterated parent molecule (60). Rakoff also synthesized a number of deuterated polyunsaturated fatty acids, *viz.*18:3(9*c*,12*c*,15*c*)-15,16-d_2, 18:3(9*c*,12*c*,15*c*)-6,6,7,7-d_4 (63), 18:3(6*c*,9*c*,12*c*)-15,15,16,16-d_4 (64), 18:4(6*c*,9*c*,12*c*,15*c*)-12,13,15,16-d_4 (65), and 20:3(11*c*,14*c*,17*c*)-3,3,4,4,8,8,9,9-d_8 (66). The structures and the configurations of these deuterated fatty acid esters were confirmed by ^{13}C NMR and mass spectrometry.

Oxygenated fatty acid esters. Tulloch (60) and his colleague Mazurek (67) have synthesized all positional isomers of hydroxystearic acid and those of ketostearic acid and have studied the ^{13}C NMR spectral properties of these closely related compounds. The presence of the hydroxy function in the alkyl chain of the fatty ester was evident from the shift of the methine carbon atom which was attached to the hydroxy group. The carbon shift of the methine carbon was found in the region of δ_C 71. The shifts of the β- and γ-methylene carbons adjacent to the hydroxy function appeared at about δ_C 37 and 25, respectively. Many of the positional isomers of hydroxystearate were identified by their spectral properties because the position of the signals in the spectrum was also affected by the carboxylate or terminal methyl group, depending on the location of the hydroxy function.

Fatty acids containing a keto function were readily identified by the appearance of a signal in the region of δ_C 210. The shifts of the β- and γ-methylene carbons adjacent to the keto function appeared at about δ_C 42 and 23, respectively (60). Bascetta and Gunstone (68) reported the ^{13}C NMR shifts for

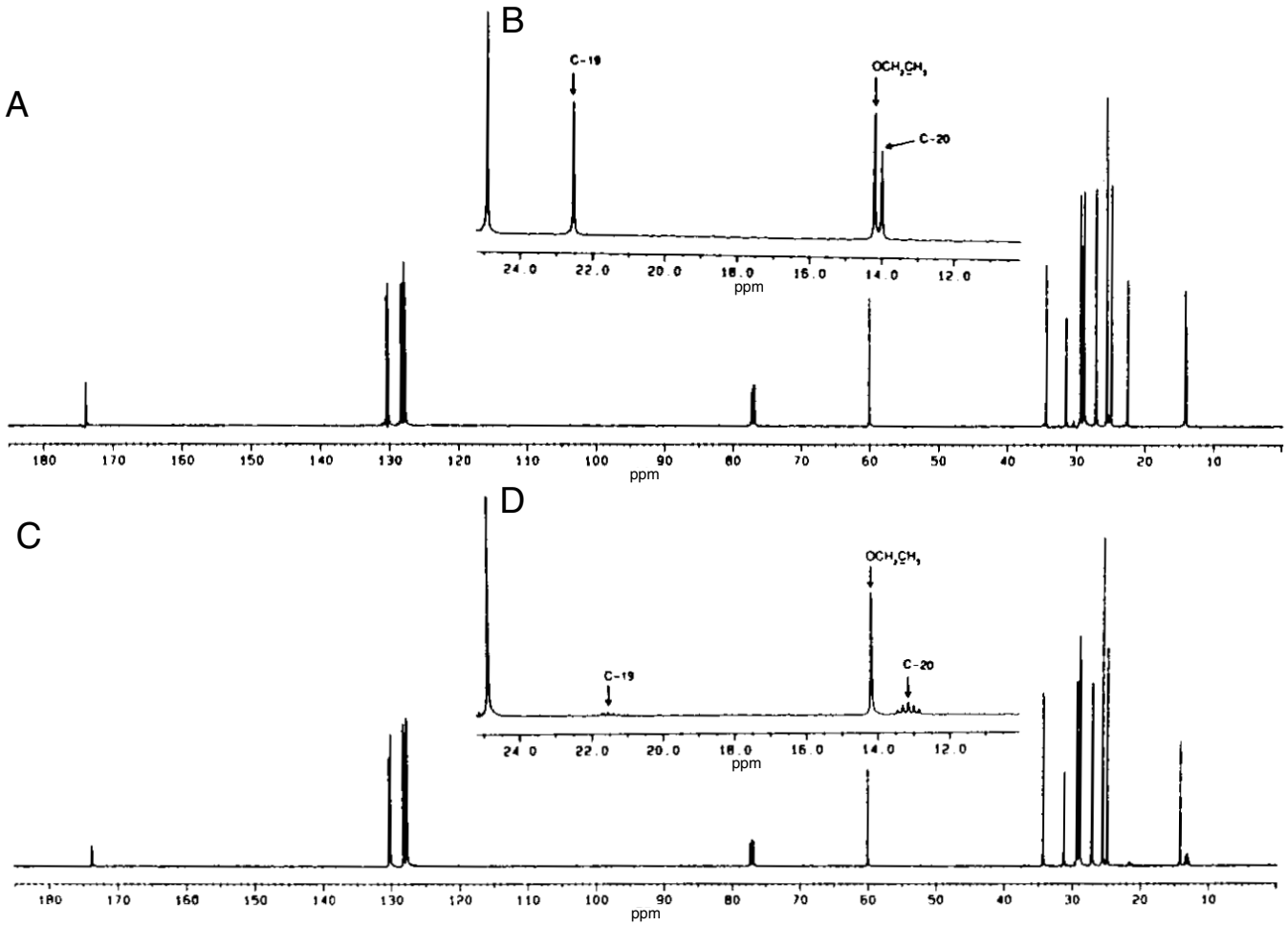


FIG. 6. ^{13}C nuclear magnetic resonance spectra of ethyl 8,11,14-eicosatrienoate: (A) nondeuterated sample, (B) expansion between 10–25 ppm, (C) deuterated sample, (D) expansion between 10–15 ppm (61).

a number of long-chain and cyclic epoxides (*cis* and *trans*, saturated and unsaturated) and of several primary alcohols and hydroperoxides. The influence of each of these functional groups on the chemical shift of nearby carbons was determined and the shift parameters reported.

A survey of the chemical literature shows conflicting assignments of the ^{13}C carbon chemical shifts for the olefinic carbon atoms of homoallylic-substituted unsaturated fatty esters. Methyl ricinoleate [methyl 12-hydroxy-18:1(9*c*)] and methyl isoricinoleate [methyl 9-hydroxy-18:1(12*c*)] are typical examples of such hydroxylated unsaturated fatty esters, which contain a homoallylic and a bis homoallylic hydroxy function in the alkyl chain, respectively. Lie Ken Jie and Cheng (69) recently used a combination of selective irradiation ^1H NMR experiments and 2D COSY (^{13}C-^1H) to determine the carbon chemical shifts of the olefinic carbon atoms. Subsequently, the ^{13}C NMR properties of a series of hydroxy, acetoxy, azido, chloro, and oxo derivatives of unsaturated fatty esters were studied (70).

Several reports have been made on the chemical hydroxylation of unsaturated fatty acids. Knothe *et al*. (71) studied the hydroxylation of oleic acid with selenium dioxide in the presence of *tert*-butylhydroperoxide. The isolated products, *viz*., 8-hydroxy-18:1(9*t*), 11-hydroxy-18:1(9*t*) and 8,11-dihydroxy-18:1(9*t*), were identified by a combination of ^1H- and ^{13}C NMR spectroscopy. Several novel allylic mono- and dihydroxy fatty compounds were synthesized from Δ^5, Δ^7, Δ^8, and Δ^{10} monounsaturated fatty acids with selenium dioxide/*tert*-butylhydroperoxide. The resonance difference of the olefinic carbon atoms was studied by ^{13}C NMR spectroscopy, which allowed the position of the unsaturated center to be located (72,73).

Four unusual hydroxy fatty acids (7-hydroxy-8,14-dimethyl-9-hexadecenoic acid, 7-hydroxy-8,16-dimethyl-9-octadecenoic acid, 7-hydroxy-8,18-dimethyl-9-eicosenoic acid, and 7-hydroxy-8,20-dimethyl-9-docosenoic acid) were identified from fungal species belonging to Ascomycetes and Basidiomycetes by the combination of NMR spectroscopic and mass spectrometric techniques. For example, the ^{13}C NMR result of 7-hydroxy-8,14-dimethyl-9-hexadecenoic acid was reported with the following critical carbon shifts: 72.1 (C-7), 28.5 (C-8), 129.8 (C-9), 129.1 (C-10), 19.8 (Me of C-14), and 22.7 (Me of C-8) (74). Filoboletic acid [8,13-dihydroxy-18:2(9*c*,11*t*)] was isolated as the antiviral principle from a

fungus belonging to the genus *Filoboletus.* The structure was elucidated by a combination of mass spectrometry and advanced techniques in ^{13}C NMR spectroscopy (including 2D COSY, HMQC, and HMBC correlation techniques), which allow the shifts of all carbon atoms to be identified (75). A cytotoxic fatty acid, 11-hydroxy-4-methyl-2,4,6-dodecatrienoic acid, was found in the extract of fermentations of the *Mucor* sp. The structure of this fatty acid was elucidated by the HMBC technique (76).

Studies of triacylglycerols. Most seed oils are composed of triacylglycerols, which contain an array of fatty acids, saturated as well as unsaturated, and distributed among the three positions of the glycerol "backbone." Fat from animals or oils from fish also appear in the form of triacylglycerols. A detailed and systematic study of saturated and unsaturated triacylglycerol molecules was conducted by Lie Ken Jie and colleagues (77–79). The ^{13}C NMR spectrum of a triacylglycerol has two characteristic signals at around δ_C 68.9 and 62.1 for the β- and α-glycerol carbon atoms, respectively. More significantly, in triacylglycerols of type AAA (where all fatty acyl chains are the same), two signals are evident for many of the carbon atoms in the acyl chain, depending on whether the chain is present in the α- (i.e,. at the 1- and 3-positions of the glycerol) or β- (i.e., 2-position of the glycerol) position. Since there are two α-chains and only one β-chain, it is easy to assign the signals on the basis of their relative intensities or sizes. For example, all acyl carbon atoms of α- and β-chains are resolved in the case of glycerol tributyrate (tributyrin), while in glycerol tridodecanoate (trilaurin) seven (*viz.,* C-1 to C-7 of the acyl chains) of the 12 carbon atoms are separated into pairs of signals each (77). The difference between each pair tends to be a constant value, which permits the signal for each of the seven carbon atoms (C-1 to C-7) to be readily identified. The chemical shifts for glycerol tributanoate and glycerol tridodecanoate are given in Table 3.

From these studies of saturated triacylglycerols of type AAA, apparently the small differences between two signals arising from carbon atoms in similar positions in the acyl chain, but not in identical chemical environments, were more reproducible than the observed shifts of the carbon atoms themselves. However, it must be pointed out that the differences in the shift values of similarly positioned carbon atoms in *short* α- and β-acyl chains (for example in the case of triacetin and tributyrin) showed significant deviations from established norms, where the acyl moieties consisted of long-chain fatty acid groups (77). Lie Ken Jie *et al.* (77) also studied the ^{13}C NMR spectra of a number of saturated triacylglycerols of type ABA (where the α-acyl chains comprised the same fatty acid and where the β-acyl chain was a different fatty acid moiety) and those of type AAB. On the whole, it was possible to identify triacylglycerols of type AAA, where the number of carbon atoms in the acyl chain did not exceed 10 carbon atoms. This technique also distinguished the mixed triacylglycerols (type ABA and AAB) from type AAA by evaluating the differences in chemical shift values of the C-1 carbon atoms of these sets of lipid molecules.

TABLE 3
^{13}C NMR Chemical Shift Values of Glycerol Tributyrate (tributyrin) and Glycerol Tridodecanoate (trilaurin) (Ref. 77)[a]

CH_2–O–CO–$(CH_2)_nCH_3$
|
CH–O–CO–$(CH_2)_nCH_3$
|
CH_2–O–CO–$(CH_2)_nCH_3$

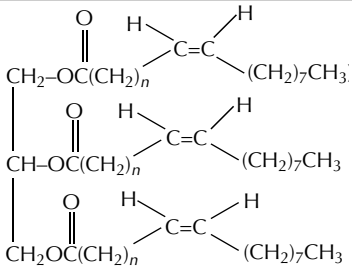
Carbon nuclei	Tributyrin (n = 2)		Trilaurin (n = 10)	
CH_2 glycerol	62.113		62.124	
CH glycerol	68.927		68.916	
	(α)	(β)	(α)	(β)
C-1	173.136	172.735	173.278	172.869
C-2	35.938	36.091	34.078	34.246
C-3	18.366	18.397	24.895	24.937
C-4	13.630	13.569	29.146	29.112
C-5			29.295	29.322
C-6			29.509	29.526
C-7			29.654	29.677
C-8			29.654	
C-9			29.379	
C-10			31.956	
C-11			22.714	
C-12			14.122	

[a]See Table 1 for abbreviation.

The ^{13}C NMR properties of a total of 22 synthetic *cis*- and *trans*-olefinic triacylglycerols of type AAA (containing isomeric positional unsaturated fatty acid homolog with the olefinic bond located between Δ^2 and Δ^{12} positions) have been studied (78). The results showed that the shifts of the glyceryl carbon atoms were only significantly affected when the unsaturated center was located at either the Δ^2 or Δ^3 position of the acyl chains. The olefinic carbon atoms in the α- and β-acyl chains were fully resolved except in two cases. From the carbon chemical shift values of the ethylenic carbons and the difference between the shift values of these nuclei in the same acyl chain, determination of most of the positions of the unsaturated center was possible. Using the signal pattern displayed by the olefinic carbon nuclei in the spectra, the positional distribution of short-chain acid moieties in the glycerol "backbone" was determined. Methylene carbon atoms in the α- and β-acyl chain, which were located close to the acyl function, were resolved up to the C-6 carbon atom. It is possible to locate the position of the unsaturated center in the acyl chains as remote as the Δ^{12} position and the positional distribution (α- or β-acyl) of the unsaturated acyl chains on the glycerol "backbone" of these triacylglycerol molecules (78). The carbon chemical shifts of triacylglycerols of type AAA containing *cis*- and *trans*-olefinic acyl groups were reproduced in Tables 4 and 5, respectively.

A large number of triacylglycerols of type AAA containing polyunsaturated (olefinic and acetylenic) acids, including mixed triacylglycerols, also were studied by Lie Ken Jie and Lam (79). From the carbon shift values of the various carbon atoms, confirmation was made of the position and geometry of the unsaturated centers in the acyl chains. Many of the similarly positioned carbon atoms, whether arising from methyl-

TABLE 4
^{13}C NMR Chemical Shift Values of Triacylglycerols of Type AAA Containing *cis*-Olefinic Acyl Groups (1–11) (Ref. 78)[a]

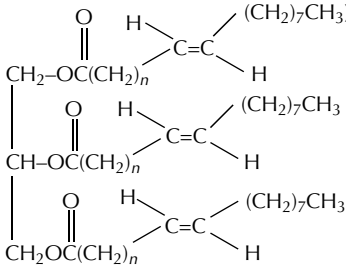


Compound	*n*	Glyceryl chain	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
1	0	61.905[a]	165.756	*118.786*[c]	*151.974*	29.192	29.358	29.274	29.457	29.039	31.892	22.696
		68.590[b]	165.233	*118.912*	*152.142*	29.247	29.390	29.283	—	—	—	—
2	1	62.334	171.513	32.708	*120.161*[d]	*133.947*	27.461	29.509	29.312[e]	29.328	29.312	31.907
		69.161	171.127	32.848	*120.171*	—	27.490	29.521		29.340	—	—
3	2	62.184	172.678	34.110	22.688	*127.014*	*131.804*	27.257	29.660	29.359	29.561	29.348
		69.003	172.284	34.241	22.699	*126.987*	*131.777*	27.276	—	29.367	—	—
4	3	62.152	173.112	33.443	24.828	26.513	*128.168*	*131.290*	27.277	29.731	29.362[e]	29.560
		68.952	172.718	33.631	24.894	26.497	*128.172*	*131.283*	—	29.743		29.567
5	4	62.141	173.144	33.946	24.503	29.189	26.832	*128.959*	*130.561*	27.285	29.772	29.374
		68.956	172.743	34.105	24.542	29.142	—	*128.970*	*130.567*	—	—	—
6	5	62.119	173.178	34.009	24.795	28.783	29.402	27.025	*129.382*	*130.249*	27.265	29.790
		68.935	172.777	34.168	24.828	28.741	29.414	—	*129.359*	*130.265*	—	—
7	6	62.114	173.197	34.029	24.851	29.057	28.936	29.595	27.157	*129.585*	*130.100*	27.260
		68.928	172.792	34.198	24.888	29.022	28.954	29.612	—	*129.558*	*130.120*	—
8	7	62.113	173.224	34.041	24.876	29.124	29.217	29.147	29.742	27.202	*129.720*	*130.014*
		68.921	172.819	34.206	24.914	29.084	29.239	29.162	29.755	—	*129.693*	*130.029*
9	8	62.110	173.216	34.048	24.883	29.147	29.416	29.293	29.364[e]	29.785	27.229	*129.780*
		68.917	172.812	34.214	24.922	29.111	29.440	29.312		29.793	—	*129.757*
10	9	62.110	173.232	34.055	24.886	29.153	29.491	29.309[e]	29.552[e]	29.331[e]	29.809	27.244
		68.912	172.827	34.221	24.926	29.115	29.511				—	—
11	10	62.102	173.190	34.052	24.894	29.159	29.525	29.325	29.643	29.598	29.381	29.817
		68.912	172.788	34.218	24.933	29.122	29.548	29.347	29.664	29.612	—	—

Compound	*n*	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20	C-21
1	0	14.117	—	—	—	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
2	1	22.697	14.119	—	—	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
3	2	31.934	22.714	14.131	—	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
4	3	29.345	31.935	22.710	14.129	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
5	4	29.573	29.363	31.949	22.722	14.135	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
6	5	29.364	29.572	29.364	31.948	22.720	14.133	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
7	6	29.803	29.367	29.572	29.367	31.952	22.721	14.135	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
8	7	27.254	29.809	29.370	29.577	29.370	31.956	22.726	14.138	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
9	8	*129.951*	27.249	29.811	29.364	29.576	29.372	31.956	22.724	14.136	—	—
		129.963	—	—	—	—	—	—	—	—	—	—
10	9	*129.822*	*129.921*	27.244	29.809	29.368	29.572	29.363	31.953	22.722	14.133	—
		129.806	*129.929*	—	—	—	—	—	—	—	—	—
11	10	27.246	*129.826*	*129.885*	27.246	29.817	29.367	29.583	29.367	31.962	22.731	14.139
		—	*129.813*	*129.892*	—	—	—	—	—	—	—	—

[a]Type AAA: all fatty acyl chains are the same. See Table 1 for other abbreviation. [a]Shift values in the α-acyl chains. [b]Shift values in the β-acyl chain. [c]Italic values denote shifts of the ethylenic carbon atoms. [d]Underlined values are obtained from 100.4 MHz spectra. [e]Signal overlapped with other signals.

TABLE 5
^{13}C NMR Chemical Shift Values of Triacylglycerols of Type AAA Containing *trans*-Olefinic Acyl Groups (12–22) (Ref. 78)[a]



Compound	*n*	Glyceryl chain	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
12	0	62.227[a]	166.138	*120.406*[c]	*150.767*	32.313	27.965	29.187	29.371	29.208	31.865	22.673
		68.960[b]	165.688	*120.474*	*151.004*	32.337	27.953	—	—	—	—	—
13	1	62.250	171.570	37.795	*120.900*	*135.319*	32.533	29.494	29.199	29.320	29.230	31.930
		69.099	171.930	37.881	*120.893*	*135.278*	32.560	29.502	—	—	—	—
14	2	62.153	172.643	34.145	27.815	*127.617*	*132.107*	32.552	29.490	29.210	29.516	29.338
		68.996	172.246	34.286	27.849	*127.586*	*132.100*	32.570	29.500	29.218	—	—
15	3	62.140	173.154	33.348	24.705	31.888	*128.674*[d]	*131.867*	32.618	29.595	29.248	29.524
		68.945	172.751	33.515	27.773	31.839	*128.678*	*131.859*	32.627	29.611	—	—
16	4	62.123	173.163	33.896	24.348	29.045	32.189	*129.436*	*131.058*	32.630	29.645	29.245
		68.923	172.763	34.054	24.391	29.004	32.204	*129.478*	—	32.639	29.654	29.256
17	5	62.111	173.184	34.016	24.749	28.618	29.251	32.387	*129.864*	*130.745*	32.637	29.673
		68.925	172.781	34.177	24.785	28.586	29.268	32.397	*129.845*	*130.752*	—	—
18	6	62.114	173.236	34.045	24.842	28.995	28.772	29.452	32.533	*130.062*	*130.589*	32.641
		68.914	172.287	34.213	24.880	28.956	28.795	29.470	—	*130.036*	*130.605*	—
19	7	62.123	173.281	34.055	24.877	29.157	29.087	28.998	29.613	32.591	*130.196*	*130.502*
		68.906	172.869	34.219	24.917	29.110	29.110	29.017	29.629	—	*130.173*	*130.517*
20	8	62.110	173.232	34.057	24.884	29.146	29.274	29.146[e]	29.366	29.662	32.627	*130.255*
		68.916	172.825	34.220	24.922	29.113	29.295		—	—	—	*130.235*
21	9	62.118	173.274	34.062	24.882	29.143	29.299	29.473[e]	29.180	29.504	29.687	32.642
		68.911	172.861	34.228	24.922	29.106	29.323		29.192	—	—	—
22	10	62.097	173.180	34.047	24.895	29.158	29.320	29.638	29.523[e]	29.219[e]	29.557	29.707
		68.913	172.780	34.213	24.934	29.119	29.343	29.657			—	—

Compound	*n*	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20	C-21
12	0	14.103	—	—	—	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
13	1	22.705	14.123	—	—	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
14	2	31.938	22.709	14.125	—	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
15	3	29.349	31.944	22.712	14.128	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
16	4	29.528	29.352	31.943	22.713	14.130	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
17	5	29.233	29.531	29.357	31.945	22.713	14.128	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
18	6	29.684	29.228	29.525	29.351	31.938	22.710	14.129	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
19	7	32.642	29.690	29.224	29.525	29.350	31.937	22.710	14.128	—	—	—
		—	—	—	—	—	—	—	—	—	—	—
20	8	*130.436*	32.649	29.670	29.228	29.539	29.366	31.950	22.720	14.131	—	—
		130.445	—	—	—	—	—	—	—	—	—	—
21	9	*130.300*	*130.407*	32.642	29.693	29.217	29.531	29.357	31.940	22.712	14.128	—
		130.284	*130.414*	—	—	—	—	—	—	—	—	—
22	10	32.659	*130.305*	*130.363*	32.659	29.707	29.232	29.557	29.383	31.962	22.730	14.139
		—	*130.293*	*130.369*	—	—	—	—	—	—	—	—

[a]See Tables 1 and 4 for abbreviations. [a]Shift values in the α-acyl chains. [b]Shift values in the β-acyl chain. [c]Italic values denote shifts of the ethylenic carbon atoms. [d]Underlined values are obtained from 100.4 MHz spectra. [e]Signal overlapped with other signals.

ene or olefinic carbon nuclei in the α- and β-acyl chains, are resolved to give characteristic pairs of signals. By making use of the differences in shift values for carbon atoms of similarly positioned carbon atoms in the α- and β-acyl chains, the assignment of the chemical shifts of the various nuclei was readily achieved. The nature (type of unsaturation and position of unsaturated centers) and distribution of the various acyl groups on the glycerol "backbone" of these mixed triacylglycerols also were determined by this technique (79).

Using the results from the systematic studies of the ^{13}C NMR properties of saturated and unsaturated triacylglycerols, Lie Ken Jie *et al.* (80,81) have extended their work to the analysis of margarines and natural seed oils. In the analysis of margarines, the technique allowed all major acyl groups (saturated, oleic, linoleic, and linolenic acids) and minor acyl components (different positional isomers of *cis*- and *trans*-long-chain unsaturated fatty acids) to be identified (80). The ^{13}C NMR analysis of the whole oil of *Biota orientalis* seeds confirm the presence of oleate, linoleate, linolenate, and also 20:4(5*c*,11*c*,14*c*,17*c*), and saturated fatty acids in the acyl groups by comparing the observed carbon shifts with previously established shift data for model triacylglycerols. This technique revealed that the saturated and 20:4 fatty acids may be distributed in one or both of the α-acyl positions, whereas oleic, linoleic, and linolenic acids were randomly acylated to the α- and β-acyl positions of the glycerol "backbone." The ^{13}C NMR analysis of carrot seed oil identified the presence of saturated (18:0), 18:1(6*c*), 18:1(9*c*), and 18:2(9*c*,12*c*). The saturated fatty acids were mainly found in the α-acyl positions. Semiquantitative assessment of the signal intensities gave the relative percentage of 18:1(6*c*) as 49.6% of the total fatty acids (81).

Similar differences in the chemical shifts of the various methylene and olefinic carbon atoms of acyl chain of triacylglycerol molecules have been reviewed (18). High-resolution ^{13}C NMR has been used to analyze other natural fats and oils, where the composition of *cis*- and *trans*-double bonds in a mixture of fat was determined (82). This technique also allowed the proportion of saturated, mono-, di-, and polyunsaturated fatty acids in vegetable and fish oils to be estimated (83–86).

The ^{13}C NMR spectra of the saturated and olefinic carbons in palm oil can be used for direct determination of the composition of the fatty acids in mole fractions of the saturated, monoene, and diene acid chains. The method was found to be more informative than the conventional iodine value which is used as a measure of the total unsaturation in the fatty acids (87). The ^{13}C NMR spectral analysis of the carbonyl carbon atoms of the acyl groups of triacylglycerols of palm oil allowed the composition of saturated, oleic, and linoleic acyl groups at the 1,3-position and at the 2-position of the glycerol moiety to be established. The report indicated that, except for the lack of differentiation of the saturated fatty acids, the ^{13}C NMR technique provided the same information as the tedious enzymatic hydrolysis *cum* fatty acid analysis (88). The chemical shift difference of the olefinic carbon atoms in the fatty acid chain of triacylglycerols in palm oil was found to be characteristic of the chain's glycerol position and can therefore be used to identify the glycerol position of an unsaturated fatty acid in the triacylglycerol molecule (89).

cis-Vaccenic acid, 18:1(11*c*), in palm oil was readily detected and identified on the basis of the chemical shift data of the carbonyl and olefinic carbons (90). The seed oil of *Sebastiana brasiliensis* (Euphorbiaceae) contained high levels (39%) of α-parinaric acid, 18:4(9*c*,11*t*,13*t*,15*c*). Complete assignment of the ^{1}H and ^{13}C NMR shifts of α-parinaric acid was carried out by two-dimensional NMR experiments (91). Cherry seed oil, from the Rosaceae family, contains about 10% of α-eleostearic acid, 18:3(9*c*,11*t*,13*t*), which was characterized by ^{1}H- and ^{13}C NMR spectral analysis (92).The seed oil of *Momordica balsamina* contains high levels of punicic acid, 18:3(9*c*,11*t*,13*c*) (50%), and α-eleostearic acid, 18:3(9*c*,11*t*,13*t*) (13%). The structure and the quantities of these conjugated fatty acids in the oil were determined by a combination of gas–liquid chromatographic and ^{13}C NMR analyses (93). Tulloch (94) studied the spectra of seven seed oils (*Punica granatum, Cucurbita palmata, Jacaranda mimosifolia, Centranthus ruber, Catalpa bignonioides, Chilopsis linearis,* and *Calendula officinalis*) and identified six C_{18} isomeric conjugated trienes by this technique.

A study of triacylglycerol species in *Santalum album* (Linn.) seed oil was carried out by comparing the results with synthetic santalbic acid, 18:2(9*a*,11*t*). The distribution of the various fatty acids, including santalbic acid, on the glycerol "backbone" was determined by the results from the ^{13}C NMR spectroscopic analysis (95). Application of the technique to hydrogenated fats led to a semiquantitative estimation of the various 18:1 isomers present (96,97). Mallet *et al.* (98) studied the ^{13}C NMR spectral differences between triolein and tripetroselinin (glycerol tri-6-*cis*-octadecenoate), and applied this method for the determination of petroselinic acid in 10 Umbelliflorae seed oils with success. The distribution of Δ^5 polyene acids, mainly 18:3(5*c*,9*c*,12*c*), in some pine seed oils between the α- and β-acyl positions by ^{13}C NMR was reported. The Δ^5 acids were present only among the α-chains (99,100). The ^{13}C NMR spectra of six seed oils containing petroselinic acid (carrot, celery, parsnip, parsley, caraway, and coriander), of aquilegia oil containing columbinic acid, 18:3(5*t*,9*c*,12*c*), and of meadowfoam oil (*Limnanthes alba*) containing other Δ^5 acids were also reported (101). Using the ^{13}C NMR signals for the C-1 and C-2 carbon atoms, it was possible to determine the proportion of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the α- and β-acyl positions of triacylglycerols. The results from the analysis of seven fish oils showed that DHA was concentrated in the β-acyl position, but EPA was more randomly distributed between the two positions (86,102).

The fatty acid composition, with emphasis to the n-3 fatty acids, of lipid extracted from the white muscle of Atlantic salmon (*Salmo salar*) was investigated. Complete assignments of the spectra of DHA and EPA were obtained from T_1 measurements and from lanthanide-induced shift studies. The

findings provide a basis for the use of ^{13}C NMR in analyzing fish lipids (45). Quantitative measurements and positional distribution of n-3 fatty acids in other fish lipids also were carried out (103,104).

The positional distribution of fatty acids on the α- or β-acyl of the glycerol backbone was assessed by investigating the carbon chemical shifts of the carbonyl and olefinic nuclei (105–108). As a result, stearic acid, for example, was found mainly distributed to the α-acyl chain of the glycerol molecule in palm and corn oil. Analysis of the positional distribution of fatty acids in butter oil was accomplished by high-resolution ^{13}C NMR method. Pfeffer *et al*. (109) showed that the predominance of butyrate was located at the α-positions of the triacylglycerols of butter oil by this technique.

The resolution of closely related triacylglycerols, such as glycerol 1,2-dilinoleate-3-linolenate (LLLn), glycerol 1,2-dilinolenate-3-linoleate (LnLLn), glycerol 1,3-dilinoleate-2-linolenate (LLnL) and glycerol 1,3-dilinolenate-2-linoleate (LnLLn), was successfully accomplished by ^{13}C NMR analysis (110). The application of ^{13}C NMR to determine the positional distribution of fatty acids on the glycerol backbone in borage and evening primrose oil has been investigated (111). Gunstone (112) studied four oils containing γ-linolenic acid (evening primrose, borage, blackcurrant, and oil of javanicus) where it is shown that the signals for C-1, C-2, and C-3 were used to determine the distribution of γ-linolenic acid between the α- and β-positions for these oils. A comparative study has been carried out for the Grignard deacylation thin-layer chromatography, high-pressure liquid chromatography, and high-resolution ^{13}C NMR for the *sn*-2 positional analysis of triacylglycerols containing γ-linolenic acid. The result showed that there is very good agreement among these methods and that the ^{13}C NMR method proved to be the easiest and most convenient in determining the *sn*-2 position for oil or triacylglycerol samples (113).

Recently the ^{13}C NMR study of cyclopropenoid triacylglycerols was reported. Full ^{13}C NMR shift assignments were made for both the sterculate and malvalate moieties in the seed oil from *Sterculia foetida.* The chain distribution was determined from quantitative NMR measurements (allowing sufficient relaxation delay to prevent saturation to improve the accuracy in integration of the signal intensities) (114). The spectra of *Vernonia galamensis* seed oil and of epoxidized palm super olein, soybean oil, and linseed oil were recorded and interpreted. The carbon shifts of the epoxy carbon atoms appeared at about δ_C 56–57. The epoxide function differed from a double bond in its influence on the chemical shifts of nearby carbon atoms (115).

^{13}C NMR spectroscopy is used in the identification of 1,3-divernoloyl glyceride, which was obtained from enzymatic synthesis from *V. galamensis* seed oil. This method also provided a quantitative approach to study the enzyme-catalyzed transformation (116). The triacylglycerols of *V. galamensis* and *Crepis alpina* seed oils were characterized from the high concentration of vernolic acid (*cis*-12,13-epoxy-*cis*-9-octadecenoic acid) and crepenynic acid (*cis*-9-octadecen-12-ynoic acid). These acids were identified by ^{1}H and ^{13}C NMR spectroscopic analyses (117). ^{13}C NMR spectroscopic analysis was used to determine quantitatively the amount of castor oil in edible and heat-abused oils. The minimal detection limits achieved for qualitative and quantitative analyses were 2.0 and 3.0%, respectively (118).

Gunstone (119) studied mono-, di-, and triacylglycerols, showing a qualitative and semiquantitative estimation of each type of ester from the results of the shifts for the three glycerol carbon atoms and for the C-1 and C-2 nuclei in each acyl chain. ^{13}C NMR analysis of derivatives of monoglycerides was employed to give a quantitative estimation of such esters (120). Further application of the quantitative method was applied to the determination of grades of virgin olive oils by determining the presence and composition of mono-, di-, and triacylglycerols in these oils (121).

There is one area where NMR technique has no rival. This involves the detailed analysis of the oil content of intact oilseeds. Schaefer and Stejskal (122) used this method to select soybeans for producing oil depleted in the less desirable linolenic acid. Other oilseeds such as castor bean, peanut, and radish seeds also have been studied (123–125).

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ARTICLE

Physicochemical Characterization of Psychosine by ^{1}H Nuclear Magnetic Resonance and Electron Microscopy

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ABSTRACT: Krabbe's disease is an autosomal recessive disease that affects the lysosomal enzyme galactosylceramidase. The storage of one of its substrates, psychosine (β-galactosylsphingosine), is thought to be responsible for the induction of pathological changes. Psychosine has a free amine group which is necessary for the mediation of its toxic effects. In the present study, the physicochemical properties of psychosine were investigated. Nuclear magnetic resonance (NMR) detected pH titration was used to determine that the amine group had a pKa of 7.18 ± 0.05. Pulsed-field gradient NMR spectroscopy was used to determine that the diffusion coefficient of 2.8 mM psychosine in D_2O at pD 4.46 or 7.04 is 1.16 ± 0.02 × 10^{-10} m^2/s or 0.77 ± 0.02 × 10^{-10} m^2/s, respectively. Negative staining electron microscopy (EM) studies of acidic and neutral solutions of psychosine also were performed. At pH 4.5, spherical structures were formed, which were relatively stable between 3, 120, and 216 h following preparation; the diameter ranged from ~14 nm at the earliest time point to ~18 nm at the last time point. The critical micelle concentration (CMC) was 1.26 mM at pH 4.0. At pH 7.1, the structures changed from spherical structures with a diameter of 15–23 nm, at the earliest time point, to a heterogeneous population of structures ranging from spherical structures, with a diameter of only a few nm, to irregularly shaped oblong structures that had one or more dimensions exceeding 100 nm. The NMR and EM data indicate that the deprotonation of the amine group causes psychosine to form aggregates that are unstable, which prevents a determination of the CMC at a neutral pH. These data indicate that molecular interactions of psychosine at the acidic pH of the lysosome, where it is normally digested, are more orderly than those at the pH of the cytoplasm or extracellular space where psychosine goes during disease.

Lipids 32, 1035–1040 (1997).

Psychosine (2-amino-3-hydroxy-4-octadecenyl-beta-D-galactopyranoside or β-galactosylsphingosine) is digested by the lysosomal enzyme galactocerebroside β-galactosidase (galactosylceramidase). In Krabbe's disease, which is an inherited autosomal disorder, this enzyme is defective and the concentration of psychosine increases 10- to 100-fold above normal levels (1,2). The inability of galactosylceramidase to metabolize psychosine led to the hypothesis that psychosine was responsible for inducing pathological changes (3). Several different mechanisms have been postulated to account for its toxic action: (i) psychosine is an inhibitor of protein kinase C (PKC), and inhibition of PKC could initiate pathological events (4); (ii) psychosine causes hemolysis to red blood cells (5), and it causes oligodendrocyte processes, but not the soma, to disintegrate (6); and (iii) psychosine can block mitochondrial enzymes (7–9). Psychosine also has been shown to inhibit cytochrome oxidase molecules facing in either direction in proteoliposomes and submitochondrial particles, suggesting that it can rapidly interact with both sides of a membrane when added externally (10), and it causes an increase in the turbidity of brain homogenates (5).

In Krabbe's disease, psychosine is exposed to environments with a pH of 4.5 or 7.4. In the lysosome, where it is normally digested, the pH is approximately 4.5. In extralysosomal spaces such as the cytoplasm or extracellular milieu, where unmetabolized psychosine goes when it escapes from lysosomes or dying cells, respectively, the pH is approximately 7.4. Psychosine has a free amine functional group which has been shown to be involved with its hemolytic activity (5) and the inhibition of PKC (4). Protonation of this amine group should markedly influence the physicochemical properties of psychosine and, as a result, direct the mechanism of its cytotoxic action.

In the present study, the physicochemical properties of psychosine were examined in acidic and neutral aqueous solutions. Nuclear magnetic resonance (NMR) detected pH titration was employed for the determination of the pKa of the psychosine amine group. In addition, pulsed-field gradient (PFG) NMR spectroscopy and electron microscopy (EM) were used to examine the propensity of psychosine to form

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Abbreviations: BPP-LED, bipolar longitudinal eddy-current delay; CMC, critical micellar concentration; EM, electron microscopy; NMR, nuclear magnetic resonance; PFG, pulsed-field gradient; PKC, protein kinase C.

aggregates at neutral and acidic pH. PFG NMR is a well-established method for the investigation of aggregation and micelle formation *in vitro* (11–15). The fundamental protonation and aggregation properties investigated in the present study may have direct bearing on the metabolism of psychosine *in vivo*.

EXPERIMENTAL PROCEDURES

Materials. β-Galactosylsphingosine (psychosine) was obtained from Sigma (St. Louis, MO). D_2O (99.9% *d*, Sigma) was used as the solvent in the NMR studies. Water purified to 18 mΩ resistance was used for the EM measurements. DCl (Cambridge Isotope Laboratories, Andover, MA) and NaOD (Isotec Inc., Miamisburg, OH) were used to adjust the pH of the psychosine solutions. All pH measurements were performed with a Fisher-Scientific (Pittsburgh, PA) Acumet 10 pH meter using a 3-mm Ingold combination microelectrode calibrated with aqueous pH buffers. The pD values reported for solutions analyzed by NMR spectroscopy have been corrected for the isotope effect using the relationship pD = pH meter reading + 0.4 (16).

NMR spectroscopy. The ^{1}H NMR spectra were measured using a Bruker 500 AM spectrometer (Billerica, MA) at temperature of 298 K. One-dimensional NMR spectra were acquired as a function of pD with a 6024 Hz spectral width and 32,764 data points. In most NMR experiments, the HOD resonance was suppressed by application of a selective saturation pulse prior to acquisition. Proton chemical shifts are reported relative to 3-(trimethylsilyl)propionic acid, 2,2,3,3-d_4 sodium salt (Aldrich, Milwaukee, WI) used as an external standard. The proton resonances were assigned using the double-quantum filtered correlated spectroscopy spectrum and by comparison with the reported assignments for psychosine and related galactocerebrosides (17,18).

Diffusion coefficient measurements. Diffusion coefficients were measured by PFG NMR spectroscopy using the bipolar longitudinal eddy-current delay (BPP-LED) pulse sequence (19). In this experiment, an LED Te is used to minimize spectral artifacts resulting from residual eddy currents. In our experiments, a Te of 15 ms was used. The spectra were measured using a Bruker 500 MHz AM spectrometer specially modified to accommodate PFG experiments. Details of the gradient instrumentation have been described previously (20). A 5-mm Bruker inverse probe with an actively shielded z-gradient coil was used. The coil constant was calibrated with β-cyclodextrin which has a diffusion coefficient of 3.2×10^{-10} m^2/s.

In BPP-LED experiments, the attenuation of the NMR resonance depends on gradient strength as shown in Equation 1:

$$I = I_0 \exp [-D(\Delta - \delta/3 - \tau/2)\gamma^2 g^2 \delta^2] \quad [1]$$

where I_0 is the intensity of the resonance in the NMR spectrum in the absence of gradient pulses, γ is the gyromagnetic ratio, Δ is the time period during which diffusion occurs, *g* and δ are the gradient amplitude and duration time, respectively, and *D* is the translational diffusion coefficient. In the BPP-LED experiments, τ is the separation of the bipolar pair of gradient pulses, which were 1.2 or 4 ms. Typically, in each pulsed-field gradient NMR experiment, a series of 15 ^{1}H spectra were collected with the BPP-LED pulse sequence as a function of gradient amplitude. In our experiments, the gradient duration time δ/2 was 1 ms, the gradient amplitude was varied from 0.024 to 0.4 Tm^{-1} and the diffusion delay time, Δ was 0.5 s.

The individual spectra were transferred to a Silicon Graphics (Mountain View, CA) Indigo work station and processed using Felix 2.30 (Molecular Simulations Inc., San Diego, CA). Following Fourier transformation, the series of free induction decays obtained as a function of gradient amplitude were processed using diffusion-ordered spectroscopy, as described previously using computer programs generously provided by Dr. Charles S. Johnson, Jr. (15,21). Diffusion coefficients were extracted using the program SPLMOD (22). The results of our experiments were fit in each case to a single diffusion coefficient.

Critical micellar concentration (CMC). CMC was determined by measuring the visible absorption (404 nm) of a dye with low aqueous solubility, coumarin 503 [(7-ethylamino)-6-methyl-4-(trifluoromethyl)-2H-1-benzopyran] (Exciton, Dayton, OH), as a function of psychosine concentration. The visible absorbance of the dye solution increases dramatically at CMC because of the increased solubility of the dye in the psychosine micelles. CMC is defined by the intersection of the straight line fits of the absorbance data for concentrations below and above CMC.

EM measurements. Solutions of 4.7 and 2.4 mM psychosine were prepared in 18 mΩ H_2O, and the pH was adjusted to 4.5 or 7.1, respectively. A lower concentration of psychosine was used for the pH 7.1 solution due to its propensity to aggregate at this pH. At 3, 120, and 216 h following preparation, the samples were mixed, and one drop of the solution was placed on a FormVar-coated 200-mesh grid (Electron Microscopy Sciences, Fort Washington, PA) for 4 min. The grids were rinsed with 5 drops of 18 mΩ water that had been pH-adjusted to match the original sample. One drop of 0.1% uranyl acetate (Electron Microscopy Sciences) solution was placed on the grid for 45 s, and the excess was removed with filter paper. The grids were allowed to dry for a minimum of 10 min, and then they were viewed and photographed with a JEOL 100CXII transmission electron microscope (Boston, MA) at 80 KV.

Additional EM studies were performed to examine the effects of concentration on the morphology and/or presence of aggregates. At pH 4.5, the concentrations were 4.71, 2.36, 1.18, 0.59 and 0.29 mM, which were examined at 185 h. At pH 7.1, the concentrations were 2.40, 1.20, 0.60, 0.30, 0.15, 0.08, 0.04 and 0.02 mM, which were examined at 205 h. In order to obtain enough material to visualize it at the lower concentrations at pH 7.1, samples were removed from the bottom of the tube without mixing. No visible material was present at pH 4.5 at any of the concentrations prepared.

RESULTS AND DISCUSSION

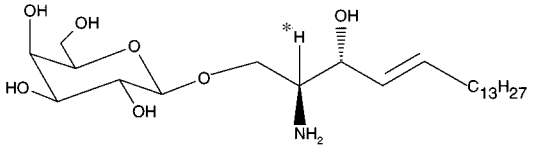
Despite the physiological and pathophysiological significance of pH for the metabolism and toxicity of psychosine (Scheme 1), the physical chemical characterization of this molecule has not received much attention. For example, we were unable to find in the literature a report of the pKa of psychosine. For hydrophobic molecules such as psychosine, a change in protonation state is often accompanied by association to form micelles or vesicles in aqueous solution. Such associative processes can sometimes be detected by a change in chemical shift or by an increase in the NMR line-width resulting from the increased correlation time associated with the aggregates. The ^{1}H NMR spectrum of 2.8 mM psychosine in D_2O solution at pD = 3.45 is shown in Figure 1A. The resonances in this spectrum are relatively sharp. However, a dramatic increase in the resonance line-width is observed as the pD is raised to 6.96 and the protonated amine group of the molecule is partially titrated as illustrated by the spectrum shown in Figure 1B. This increase in line-width probably results from an increase in the average molecular correlation time, suggesting that the neutral form of psychosine forms larger aggregates than the protonated form, a phenomenon common among surfactants and other sphingolipids (23).

The chemical shifts of several of the psychosine ^{1}H NMR resonances are somewhat sensitive to solution pH. However, the most significant pH-dependent change in chemical shift occurs for the proton bound to the same carbon atom as the amine group of psychosine (indicated by an asterisk in the structure drawn in Scheme 1). The resonance due to this proton is marked with an arrow in the spectra shown in Figure 1. In order to calculate the pKa of the amine group, the chemical shift of this proton was measured over a wide pH range as shown by the data points in Figure 2.

Since proton exchange kinetics are generally fast on the NMR time-scale, separate resonances for the amine base and its conjugate acid are not detected. Instead, a single exchange-averaged resonance is detected for this proton that has a chemical shift which is a weighted average of the protonated and deprotonated forms of the molecule. Therefore, in pH regions where a mixture of the protonated and deprotonated species is in equilibrium, the observed chemical shift (δ_{obs}) of the nuclei under examination is given by Equation 2:

$$\delta_{obs} = f_{HA}\,\delta_{HA} + f_{A-}\,\delta_{A-} \qquad [2]$$

where f_{HA} and f_{A-} are the fractional populations and δ_{HA} and δ_{A-} are the chemical shifts of the protonated and deprotonated species. Using Equation 2 and the relationship $f_{HA} + f_{A-} = 1$, the fractional populations can be expressed in terms of δ_{obs}, δ_{HA} and δ_{A-} and directly incorporated into the Henderson-Hasselbalch equation.



SCHEME 1

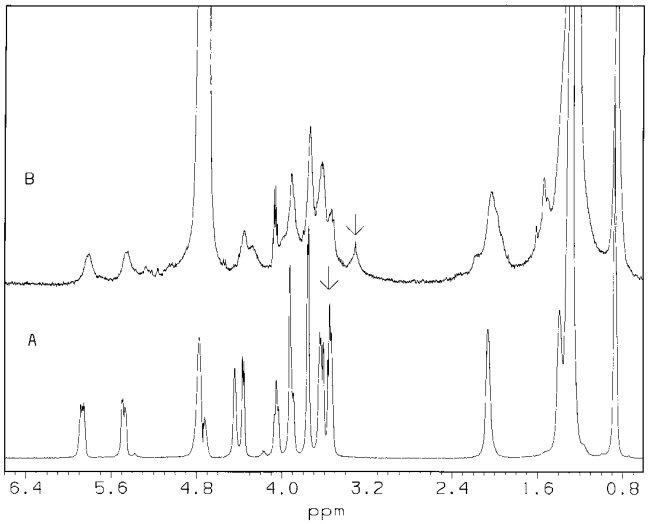


FIG. 1. The ^{1}H nuclear magnetic resonance spectrum of psychosine in D_2O (A) at pD 3.45 and (B) at pD 6.96. The arrows mark the resonance due to the proton indicated by the asterisk in Scheme 1.

A nonlinear least-squares fitting routine was used to calculate the group K_a using the above relationships. The single parameter nonlinear least-squares fit for psychosine in D_2O is shown by the solid line drawn through the data points in Figure 2. The pKa of the protonated amine group of psychosine was calculated as 7.18 ± 0.05 using this method. As described in the subsequent sections, the aggregation of psychosine, especially at higher pH, may introduce some error into the measured pKa. However, the measured pKa is in good agreement with that reported for related molecules such as sphingosine (23).

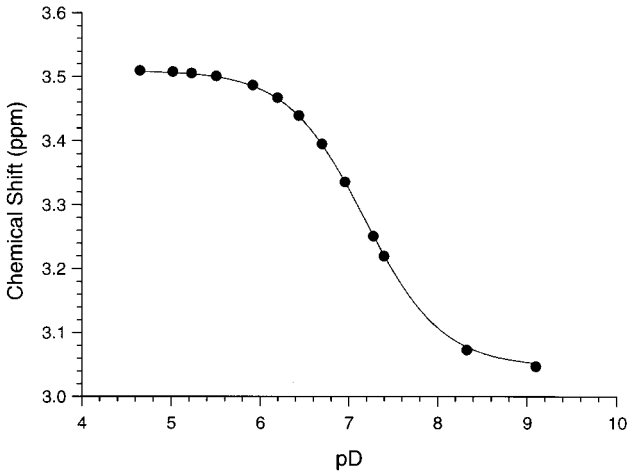


FIG. 2. The pH dependence of the nuclear magnetic resonance chemical shift of the proton adjacent to the psychosine NH group. The solid line drawn through the experimental data is the result of a nonlinear least-squares fit of the monoprotic deprotonation model to the experimental data.

Previous studies that examined the inhibition of PKC (4,6) or mitochondrial enzymes (7–9) by psychosine utilized assay conditions with a pH ranging from 7.4–7.5. Thus, the amine group on psychosine would have been mostly deprotonated (62%) in these assays. Since the free amine group has been shown to be essential for PKC inhibition (4), the charge on the amine group could be essential for the inhibition properties of psychosine. If the protonated state is the active state, then the actual concentration required for inhibition is significantly less than that reported, which represented the total amount of material added to the assays.

NMR diffusion measurements. The association of psychosine to form organized structures, such as micelles, also can be detected by monitoring the translational diffusion coefficient of the molecule in solution. Diffusion coefficients measured with pulsed-field gradient NMR spectroscopy have been employed in the study of a variety of surfactants and phospholipids (14,15,24,25). The diffusion coefficient determined for 2.8 mM psychosine in D_2O solution at pD 4.46 is $1.16 \pm 0.02 \times 10^{-10}$ m^2/s. To evaluate whether association occurs at this concentration even when psychosine is protonated, the diffusion coefficient of a 0.10 mM solution of psychosine was measured at this pD. The diffusion coefficient of psychosine in this dilute solution, $4.06 \pm 0.26 \times 10^{-10}$ m^2/s, probably reflects the diffusion coefficient of the monomer. The higher error obtained in this measurement can be attributed to the lower signal-to-noise ratio of the NMR spectra of the more dilute solution. The diffusion coefficient determined for a pD 7.04 solution of 2.8 mM psychosine, $0.77 \pm 0.02 \times 10^{-10}$ m^2/s, indicates further association of the psychosine as the fraction of the protonated form of the molecule is decreased. The pH dependence of the psychosine diffusion coefficients parallels the increase in line-width observed in the proton NMR spectrum. Although it is not possible to determine the number or shape of psychosine aggregates from the NMR experiments, the good fit of the diffusion results to a single diffusion coefficient in each case suggests fast exchange between the aggregate species.

EM and CMC measurements. The size and shape of psychosine aggregates at 4.7 mM were measured as a function of pH and time. At pH 4.5, spherical aggregates with a diameter of ~14 nm were observed at 3 (Fig. 3A) and 120 h (Fig. 3B). By 216 h, some aggregates increased in size to ~18 nm (Fig. 3C). Solutions at pH 4.5 remained clear for several months following preparation.

Additional studies were performed at 185 h to examine the function of concentration on aggregate formation at pH 4.51. Spherical aggregates were observed at 2.36 and 1.18 mM, but the density of structures was much less in the latter solution. There were no spherical aggregates at 0.59 or 0.29 mM. These findings are in good agreement with the CMC, which was found to be 1.26 mM at pH 4.0.

Increasing the pH to 7.1 dramatically affected the stability of psychosine aggregates at 2.4 mM. At 3 h, a range of spherical structures with a diameter of 15–23 nm was detected (Fig. 3D). At 120 (Fig. 3E) and 216 h (Fig. 3F,3G), a heterogeneous population of structures was observed. Some aggregates were only a few nm in diameter, while other aggregates had a diameter in excess of 100 nm with a spherical or irregular shape. Solutions at pH 7.1 eventually became turbid and remained that way for several months following preparation.

Additional studies were performed at 205 h to examine the function of concentration on aggregate formation at pH 7.15. Pleomorphic structures, which were similar to those structures depicted in Figure 3F and 3G, were observed at the following concentrations: 2.40, 1.20, 0.60, 0.30, 0.15, 0.08, and 0.04 mM. The appearance of the large structures at concentrations of 0.30 mM and lower was generally limited to dense, round structures. Structures were not observed at a concentration of 0.02 mM.

Attempts to determine the CMC at pH 7.0 were unsuccessful with coumarin 503 because a definitive break was not observed in the absorbance data for coumarin 503 as a function of psychosine concentration, although the solubility of the dye did increase concomitantly with the psychosine concentration.

Data from both NMR and EM studies indicate that psychosine forms larger aggregates at a neutral pH compared to an acidic pH, and EM studies indicate that aggregates at a neutral pH form at a lower concentration and increase in size with time. Since the pKa of the free amine group was determined to be 7.18 ± 0.05, the greater propensity to form aggregates at pH 7.1 is likely due to the deprotonation of this amine group. Also, the protonated state of this amine group is likely responsible for the decreased stability of the aggregate structures over time. It is likely that in the protonated state, the amine group is lifted above the hydrophobic layer formed by the carbon chains. This displacement of the amine group could enable the acyl chains to achieve an orderly array that is very stable. The displacement of the amine group also would extend galactose further away from the hydrophobic layer than if the amine were embedded in the hydrophobic region. This greater extension of galactose could be an important component of the enzymatic reaction by galactosylceramidase, which is the lysosomal enzyme that removes the galactose from psychosine. Galactosylceramidase has an optimal pH of 4.2–4.5 (26,27), and 99.8% of psychosine is protonated at pH 4.5. When the amine group is deprotonated, it is probably associated with the hydrophobic aspects of the aggregates. The interaction of the amine group with neighboring hydrophobic regions of adjacent molecules could result in a less orderly packing, which could account for the greater instability of the aggregates at pH 7.1. Although the deprotonated state of sphingosine (psychosine without galactose) enables it to traverse membranes (23), deprotonated psychosine would be less likely to cross membranes due to the galactose moiety.

Previous studies examining the disruption of cellular and mitochondrial membranes by psychosine (5,6,7,28) have performed assays at pH 7.4–7.5. Thus, the amine group on psychosine would mostly be in the deprotonated state in these assays. This finding suggests that the deprotonated state desta-

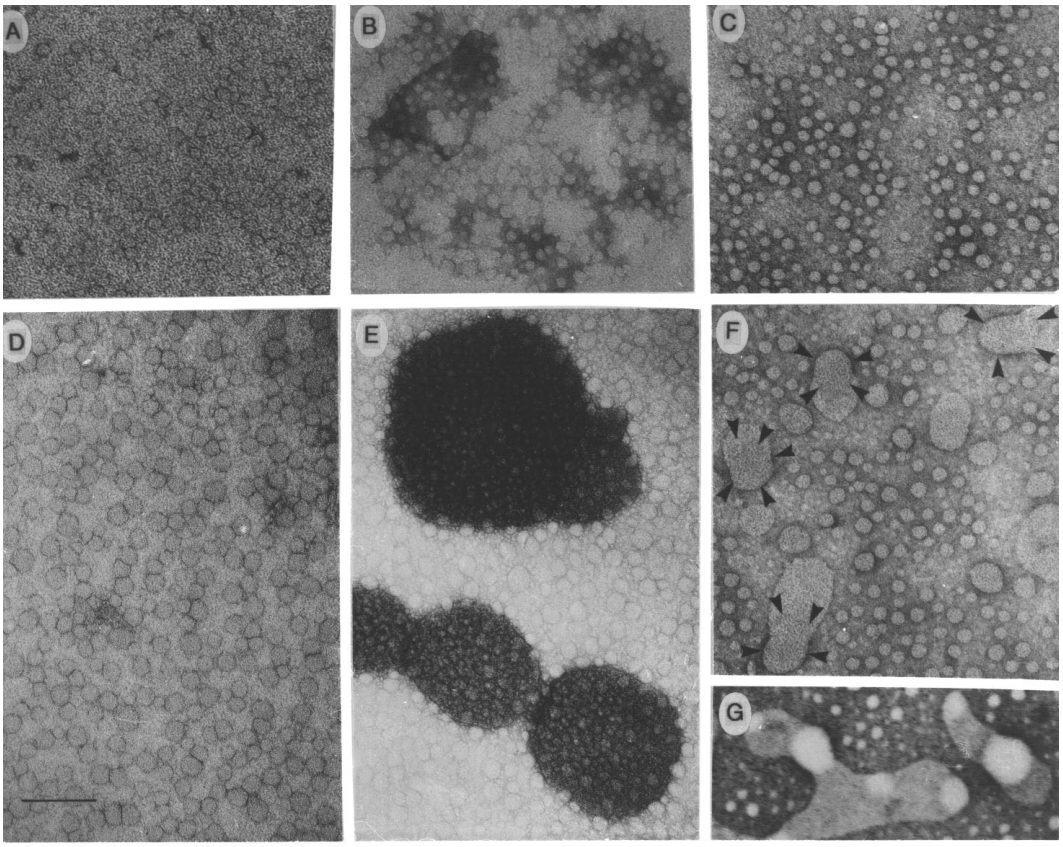


FIG. 3. Psychosine was prepared at pH 4.5 (A–C) or 7.1 (D–G) and processed for negative staining at 3, 120, and 216 h. (A,B) Spherical structures with an approximate diameter of 14 nm were observed in a solution with a pH of 4.5 at 3 h (A) and 120 h (B). Some of the structures had a slightly smaller or larger diameter. (C) Spherical structures with a diameter of ~18 nm or smaller were observed at pH 4.5, 216 h. (D) Spherical structures generally ranging in diameter from 15–23 nm were observed at pH 7.1, 3 h. Some smaller structures also were observed. (E) Two groups of structures were observed at pH 7.1, 120 h—one group had spherical structures with a diameter of ~2–~23 nm, and a second group had large, round structures that could be in excess of 100 nm in diameter. This second group of structures is seen as the intensely stained, large structures. (F,G) Structures ranging in size from a few nm to 100 or more nm were observed at pH 7.1, 216 h. Those structures with a smaller diameter generally had a round appearance, while the larger structures had an oval or irregular oblong shape. Within these larger structures, there was generally a region that had a more dense appearance than the rest of the structure. In F, the dense regions are marked by the arrowheads, and in G they appear as the white structures within the gray oblong structures. Bar = 100 nm.

bilizes and/or disrupts the organization of cellular membranes.

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Synthesis and Nuclear Magnetic Resonance Properties of All Geometrical Isomers of Conjugated Linoleic Acids

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ABSTRACT: Pure geometric isomers of conjugated linoleic acid were prepared from castor oil as the primary starting material. Methyl octadeca-9*Z*,11*E*-dienoate (**2**) and methyl octadeca-9*Z*,11*Z*-dienoate (**4**) were obtained by zinc reduction of methyl santalbate (**1**, methyl octadec-11*E*-en-9-ynoate) and methyl octadec-11*Z*-en-9-ynoate (**3**), respectively, as the key intermediates. Methyl octadeca-9*E*,11*E*-dienoate (**8**) and methyl octadeca-9*E*,11*Z*-dienoate (**9**) were prepared by demesylation of the mesyloxy derivative of methyl ricinelaidate (**6**, methyl 12-hydroxy-octadec-9*E*-enoate). A study of the nuclear magnetic resonance spectral properties was carried out, and the shifts of the olefinic carbon atoms of 18:2(9*Z*,11*E*) (**2**) and 18:2(9*E*,11*Z*) (**9**) were readily identified by a combination of incredible natural abundance double quantum transfer experiment, heteronuclear multiple bond correlation, and ^{1}H-^{13}C correlation spectroscopy correlation techniques. Doubts remain in the absolute identification of the individual olefinic carbon atoms of the 18:2(9*Z*,11*Z*) (**4**) and 18:2(9*E*,11*E*) (**8**), except the fact that the shifts of the "inner" (C-10 and C-11) and "outer" (C-9 and C-12) positioned olefinic carbon atoms of the conjugated diene system are distinguishable.

Lipids 32, 1041–1044 (1997).

There is considerable interest in conjugated linoleic acids (CLA), a mixture of geometric isomers of 18:2(9,11), which show unique biological properties. These fatty acids are recognized as special nutrients that function to regulate energy retention and protein metabolism in animals (1). CLA have been identified as potent anticarcinogens in a number of model systems (2–8). For example, CLA inhibit benzo(α)-pyrene-induced stomach neoplasia in the mouse (3). The antioxidant activity of this group of conjugated unsaturated fatty acids has been offered as a possible explanation for its anticarcinogenic effects (3,5,9). It also has been suggested that the antioxidant properties of CLA cause the reduction of atherosclerosis in CLA-fed rabbits (10). The anticancer properties of CLA may be related to products formed by autoxidation of the substrates. There is speculation that CLA may cyclize to form furan fatty acids, as the autoxidized products of furan fatty acids have been shown to inhibit urease enzyme activities (11).

In order to study the physical and biological properties of each individual geometric isomer of CLA in detail, we report in this paper the synthesis and the nuclear magnetic resonance (NMR) properties of each individual geometric isomer of CLA. Mixtures of the geometric isomers of CLA have been prepared by alkali isomerization of linoleic acid (12,13). CLA isomers were also obtained by the dehydration of methyl ricinoleate on heating *in vacuo* in the presence of $KHSO_4$ (14). Dehydration of ricinelaidic acid at high heat under vacuum formed a polyester, which on pyrolysis afforded the 18:2(9*E*,11*E*) isomer (15). Another method involved the mesylation of methyl ricinoleate, which was followed by base-catalyzed elimination of the mesyloxy function to yield CLA in good yield (16). Many of the abovementioned procedures give rise to mixtures of the conjugated dienoic acids. Our objective in this project was to produce the four geometric isomers of 18:2(9,11) in the pure form and to study their NMR properties.

RESULTS AND DISCUSSION

In planning the synthesis of the individual geometric isomers of CLA, we considered castor oil (a ready source of ricinoleic acid, 12-hydroxy-octadec-9*Z*-enoic acid) as an appropriate starting material. The procedure of Gunstone and Said (16), of demesylation of the mesyloxy derivative of methyl ricinoleate with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), appeared very suitable for the preparation of 18:2(9*Z*,11*E*), as elimination of the mesyloxy group favored the *E*-olefinic bond across the C-11/C-12 carbon atoms of the alkyl chain. However, this approach could not be extended to the production of 18:2(9*Z*,11*Z*) from the same mesyloxy derivative of methyl ricinoleate, as this isomer would constitute a minor component and would be difficult to isolate from the major product [18:2(9*Z*,11*E*)]. To circumvent this problem, methyl santalbate (**1**, methyl octadec-11*E*-en-9-ynoate) and its *Z*-isomer (**3**, methyl octadec-11*Z*-en-9-ynoate) were considered as alternative key intermediates for the synthesis of

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Abbreviations: INADEQUATE, incredible natural abundance double quantum transfer experiment; ^{13}C NMR, carbon-13 nuclear magnetic resonance spectroscopy; CLA, conjugated linoleic acid; COSY, correlation spectroscopy; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; HMBC, heteronuclear multiple bond correlation; ^{1}H NMR, proton nuclear magnetic resonance; IR, infrared spectroscopy; NMR, nuclear magnetic resonance; UV, ultraviolet spectroscopy.

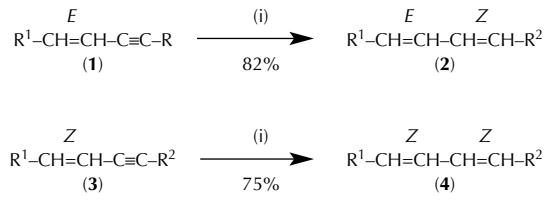
18:2(9*Z*,11*E*) and 18:2(9*Z*,11*Z*), respectively. The procedures for the synthesis of methyl santalbate (**1**) and its *Z*-isomer (**3**) from ricinoleic acid were recently reported (17). Thus, with the olefinic bond in the desired configuration and position in the alkyl chain of the enyne intermediate, zinc reduction (18) of the enyne system would furnish the requisite geometric isomers as presented in Scheme 1.

In this project the results showed that zinc in aqueous *n*-propanol was highly selective in the reduction of the acetylenic bond of the enyne intermediates to the corresponding *Z*-olefinic bond. Any unreacted enyne substrate was readily removed by treatment of the isolated product with mercury(II) sulfate in methanol, which converted the enyne intermediate to the corresponding keto-ene derivative. The latter was then separated by silica column chromatography (19). Thus, by using methyl santalbate (**1**) and its *Z*-isomer (**3**) as intermediates, we were able to obtain 18:2(9*Z*,11*E*) (**2**) and 18:2(9*Z*,11*Z*) (**4**), respectively, in the pure form.

To obtain the 18:2(9*E*,11*E*) and 18:2(9*E*,11*Z*) isomers, the procedure of Gunstone and Said (16) was adopted. Methyl ricinoleate (**5**) was first isomerized (using *p*-toluenesulfinic acid) to methyl ricinelaidate (**6**, methyl 12-hydroxy-octadec-9*E*-enoate). Demesylation of the mesyloxy derivative (**7**) of methyl ricinelaidate with DBU furnished 18:2(9*E*,11*E*) (**8**) in 76% yield and 18:2(9*E*,11*Z*) (**9**) (15%) (Scheme 2). The free acid of 18:2(9*E*,11*E*) (**8**) was readily isolated by crystallization from ethanol. The alcoholic mother liquor contained a high proportion of the 18:2(9*E*,11*Z*) isomer, which was subsequently purified by urea fractionation. This approach allowed the production of pure 18:2(9*E*,11*E*) (**8**) and 18:2(9*E*,11*Z*) (**9**), despite the fact that the latter isomer could be regarded as a side-product rather than a main product.

To assign the chemical shifts of the carbon atoms of the conjugated diene system of each CLA isomer, it was necessary to conduct INADEQUATE (incredible natural abundance double quantum transfer experiment), HMBC (heteronuclear multiple bond correlation), and two-dimensional ^{1}H-^{13}C correlation spectroscopy (COSY) correlation techniques on the carbon signals of the diene system of the *E*,*Z*-isomers. The results of these experiments for the CLA isomers are summarized in Table 1.

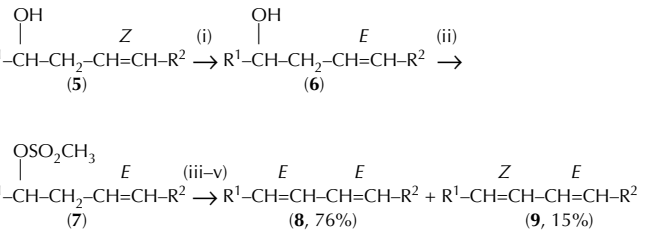
The ^{1}H NMR spectrum of 18:2(9*Z*,11*E*) (**2**) showed four distinct signals for the four olefinic protons. There appeared



R^1 = $CH_3(CH_2)_5$; R^2 = $(CH_2)_7COOCH_3$

Reagents and conditions: (i) zinc, *n*-propanol, reflux, 10 h.

SCHEME 1



R^1 = $CH_3(CH_2)_5$; R^2 = $(CH_2)_7COOCH_3$

Reagents and conditions: (i) *p*-toluenesulfinic acid, dioxane, reflux, 1 h; (ii) methane sulfonyl chloride, triethylamine, dichloromethane; (iii) 1,8-diazabicyclo[5.4.0]undec-7-ene, dimethylsulfoxide, reflux, 12 h; (iv) crystallization from ethanol and urea fractionation of mother liquor; (v) BF_3-methanol, reflux.

SCHEME 2

two multiplets (δ_H 5.32 and 5.65) and two triplets (δ_H 5.82, J = 5.4 Hz and 6.24, J = 12.9 Hz), which correspond to the shifts of the protons of the "outer" (9-*H*, 12-*H*) and "inner" (10-*H*, 11-*H*) olefinic protons of the diene system, respectively. These signals were confirmed by ^{1}H-^{13}C COSY correlation spectral analysis. From the ^{13}C NMR spectrum, the carbon signals of the methylene groups adjacent to the diene system were readily recognized, as the signals of the C-8 carbon atom (adjacent to the *Z*-olefinic bond) appeared at δ_C 27.66, while that of C-13 (adjacent to the *E*-olefinic bond) was found at δ_C 32.92. These results agreed with values reported by Bus *et al.* (20). From the INADEQUATE and HMBC spectra, it was possible to interconnect the signal at δ_C 32.92 to that at δ_C 134.76; δ_C 134.76 to δ_C 125.58; δ_C 125.58 to δ_C 128.71; and δ_C 27.66 to δ_C 129.89, while the latter signal was also

TABLE 1
^{13}C Nuclear Magnetic Resonance Chemical Shift Values of Conjugated Linoleic Acid Ester Isomers[a]

Carbon nucleus	Isomer 18:2(9*Z*,11*E*) (**2**)	18:2(9*E*,11*Z*) (**9**)	18:2(9*E*,11*E*) (**8**)	18:2(9*Z*,11*Z*) (**4**)
C-1	174.32	174.34	174.22	174.27
C-2	34.10	34.10	34.09	34.10
C-3	24.95	24.95	24.98	24.97
C-4	29.06	28.97	29.04	29.14
C-5/C-6/C-7	29.12–29.67	29.13–29.45	29.14–29.77	29.11–29.60
C-8	27.66	32.86	32.61[a]	27.46
C-9	129.89	134.51	132.16[b]	131.87[d]
C-10	128.71	125.72	130.37[c]	123.58[e]
C-11	125.58	128.57	130.51[c]	123.72[e]
C-12	134.76	130.17	132.43[b]	132.14[d]
C-13	32.92	27.72	32.68[a]	27.54
C-14	29.41	29.73	29.40	29.68
C-15	28.95	28.97	28.97	29.04
C-16	31.77	31.77	31.82	31.81
C-17	22.65	22.65	22.68	22.69
C-18	14.12	14.12	14.13	14.13
$COOCH_3$	51.44	51.45	51.39	51.42

[a]a-a, b-b, c-c, d-d, e-e interchangeable.

connected to δ_C 128.71. From these connections, it was possible to identify unequivocally the shifts of the diene carbon atoms of 18:2(9*Z*,11*E*) (**2**) as: δ_C 129.89 (C-9), 128.71 (C-10), 125.58 (C-11), and 134.76 (C-12). This result appeared to be contrary to values reported by Bus *et al*. (20), who described their assignments as: δ_C 130.00 (C-9), 125.75 (C-10), 128.90 (C-11), and 134.80 (C-12). However, from the NMR correlation techniques applied, we are of the opinion that the values for the C-10 and C-11 by Bus *et al*. (20) were erroneously assigned.

By using a combination of similar NMR techniques (INADEQUATE, HMBC, and COSY), the assignment of the signals of the various critical carbon atoms of 18:2(9*E*,11*Z*) (**9**) was determined as: δ_C 32.86 (C-8), 134.51 (C-9), 125.72 (C-10), 128.57 (C-11), 130.17 (C-12), and 27.72 (C-13). The ^{1}H NMR spectral analysis showed the signals of the olefinic protons at δ_H 5.30 (*m*, 12-*H*), 5.66 (*t*, *J* = 5.4 Hz, 11-*H*), 5.93 (*m*, 9-*H*) and 6.24 (*t*, *J* = 12.9 Hz, 10-*H*).

The ^{1}H NMR spectrum of 18:2(9*Z*,11*Z*) (**4**) showed a multiplet at δ_H 5.40 for the "outer" positioned protons of the diene system (9-*H*, 12-*H*) and a double doublet at δ_H 6.22 for the "inner" positioned protons (10-*H*, 11-*H*). The presence of a *Z*,*Z*-diene system was clear from signals in the ^{13}C NMR spectrum at δ_C 27.46 and 27.64 corresponding to the C-8 and C-13 carbon atoms, respectively. However, it was not possible to differentiate these two nuclei. A clear connection was established from the INADEQUATE spectrum, which confirmed the link of the signals at δ_C 27.46 and 27.54 to δ_C 131.87 and 132.14. However, it was difficult to assign these signals to individual carbon atoms, except that the olefinic carbon atoms were correlated with the proton signal of the "outer" position protons (C-9, C-12) of the diene system as observed from the ^{1}H-^{13}C COSY correlation spectrum. The two remaining olefinic carbon signals, δ_C 123.58 and 123.72, were correlated to the proton signal of the "inner" positioned protons of the diene system (C-10, C-11). Difficulties therefore remain for the assignment of these closely related olefinic carbon atoms.

The signals of the olefinic protons in 18:2(9*E*,11*E*) (**8**) showed the signals of the "inner" positioned olefinic protons (10-*H*, 11-*H*) in a slightly more upfield position (at δ_H 5.96) than those of the similarly positioned protons of the 18:2(9*Z*,11*Z*) isomer (**4**). The *E*,*E*-diene nature of the conjugated system was evident from the appearance of two signals at δ_C 32.61 and 32.68 for the shifts of the methylene carbon atoms (C-8, C-13) adjacent to the diene system. Similar to the *Z*,*Z*-diene isomer (**4**), it was not possible to differentiate with certainty each of the carbon atoms of the olefinic system of the *E*,*E*-diene isomer (**8**), except that the "inner" and "outer"positioned olefinic carbon atoms appeared at δ_C 130.37 and 130.51 (C-10, C-11) and 132.16 and 132.43 (C-9, C-12), respectively.

From these results we conclude that specific isomers of CLA can be obtained by partial synthesis from castor oil and that the *E*,*Z*-, *Z*,*E*-, *E*,*E*-, and *Z*,*Z*-isomers can be characterized in sufficient detail by a combination of NMR techniques.

EXPERIMENTAL PROCEDURES

Methyl octadec-11*E*-en-9-ynoate (**1**) and octadec-11*Z*-en-9-ynoate (**3**) were prepared from castor oil by the method described elsewhere (17). Infrared (IR) spectra were measured on a Shimadzu model IR-470 spectrophotometer (Shimadzu Corp., Kyoto, Japan) on neat samples placed between NaCl discs. Ultraviolet (UV) spectra were recorded on a Perkin Elmer model Lambda 19 spectrophotometer (Perkin Elmer, Inc., Norwalk, CT), NMR spectra were recorded on a Bruker Avance DPX_{300} (300 MHz) Fourier transform NMR spectrometer (Bruker, Fallanden, Switzerland) from solutions in deuteriochloroform ($CDCl_3$) (0.2–0.3 mM) with tetramethylsilane as the internal reference standard. Chemical shifts are given in δ-values in ppm downfield from tetramethylsilane ($\delta_{TMS} = 0$).

*Preparation of methyl octadeca-9Z,11E-dienoate (**2**).* A mixture of methyl octadec-11*E*-en-9-ynoate (**1**, 4.9 g, 16.7 mmol), aqueous *n*-propanol (120 mL, 50%, vol/vol), and zinc powder (75 g) was refluxed for 10 h. The cooled reaction mixture was filtered and the filtrate diluted with water (150 mL). The aqueous filtrate was extracted with diethyl ether (2 × 100 mL). The ethereal extract was successively washed with dilute HCl (2 M, 75 mL) then water (50 mL) and dried over anhydrous sodium sulfate. The filtrate was evaporated to give methyl octadeca-9*Z*,11*E*-dienoate (**2**, 4.0 g, 82%). IR (cm^{-1}) 2927, 2855, 1743 (C=O, str., ester), 1436, 1171, 982, and 947 (*trans*-unsaturation); UV_{max} 232 nm; ^{1}H NMR(δ_H) 0.88 (*t*, *J* = 6 Hz, 3H, CH_3), 1.25–1.43 (*m*, 18H, CH_2), 2.00–2.10 (*m*, 4H, 8-H_2 and 13-H_2), 2.29 (*t*, *J* = 7 Hz, 2H, 2-H_2), 3.66 (*s*, 3H, COOCH_3), 5.32 (*m*, 1H, 9-*H*), 5.65 (*m*, 1H, 12-*H*), 5.82 (*t*, *J* = 5.4 Hz, 1H, 10-*H*), and 6.24 (*t*, *J* = 12.9 Hz, 1H, 11-*H*).

*Preparation of methyl octadeca-9Z,11Z-dienoate (**4**).* Zinc reduction of methyl octadec-11-*Z*-en-9-ynoate (**3**) in aqueous *n*-propanol was conducted by a similar procedure to that described above. Unreacted substrate (about 14% by gas chromatography analysis) in the isolated product was removed by treating the mixture with mercuric sulfate in anhydrous methanol which allowed hydration of triple bond to give the corresponding keto derivatives (19). Silica gel column chromatographic purification using a mixture of *n*-hexane/diethyl ether, 95:5, vol/vol as eluent gave pure methyl octadeca-9*Z*,11*Z*-dienoate (**4**, 75%). IR (cm^{-1}) 2930, 2856, 1742 (C=O, str. ester), 1435, 1171, and 757; UV_{max} 238 nm, ^{1}H NMR (δ_H) 0.88 (*t*, *J* = 6 Hz, 3H, CH_3), 1.18–1.51 (*m*, 18H, CH_2), 2.15 (*m*, 4H, 8-H_2 and 13-H_2), 2.3 (*t*, *J* = 7 Hz, 2H, 2-H_2), 3.65 (*s*, 3H, COOCH_3), 5.40 (*m*, 2H, 9-*H* and 12-*H*), and 6.22 (*dd*, *J* = 8.1 Hz, 2H, 10-*H* and 11-*H*).

*Preparation of methyl octadeca-9E,11E-dienoate (**8**) and octadeca-9E,11Z-dienoate (**9**).* Methyl 12-mesyloxy-octadec-9*E*-enoate (**7**) was prepared according to the method described earlier (17,21). Methyl 12-mesyloxy-octadeca-9*E*-enoate (**7**, 2.0 g, 5.1 mmol) was refluxed with DBU in dimethylsulfoxide (15 mL) for 12 h. The reaction mixture was passed through a dry column of silica (30 g), and the products were isolated by eluting with a mixture of *n*-hexane/di-

ethyl ether (9:1, vol/vol, 400 mL). The recovered products (1.42 g) were treated with ethanolic KOH (5%, 20 mL) at room temperature for 1 h. Water (50 mL) was added and the aqueous solution acidified with dilute HCl (1 M, 20 mL). The reaction mixture was extracted with diethyl ether (3 × 40 mL). The ethereal extract was washed with water (2 × 20 mL) and dried over anhydrous sodium sulfate. The filtrate was evaporated to give a residue (1.3 g). The residue was recrystallized from ethanol (3 × 20 mL) to yield pure octadeca-9*E*,11*E*-dienoic acid (1.0 g, 76%). The latter was esterified to methyl octadeca-9*E*,11*E*-dienoate (**8**) using borontrifluoride methanol complex. IR (cm^{-1}) 1740 (C=O, str. ester) and 988 (C=C, *trans*); UV_{max} 231; ^1H NMR (δ_H) 0.88 (*t*, *J* = 6 Hz, 3H, CH_3), 1.08–1.79 (*m*, 14H, CH_2), 1.61 (*m*, 4H, 7-H_2 and 14-H_2), 2.00–2.07 (*m*, 4H, 8-H_2 and 13-H_2), 2.29 (*t*, *J* = 7 Hz, 2H, 2-H_2), 3.65 (*s*, 3H, $COOCH_3$), and 5.56 (*m*, 2H, 9-*H* and 12-*H*) and 5.96 (*m*, 2H, 10-*H* and 11-*H*).

The mother liquor from the above recrystallization procedure was diluted with water (50 mL) and extracted with diethyl ether (30 mL) and dried over sodium sulfate. The filtrate was evaporated, and the residue (0.23 g) was crystallized with urea (0.7 g) in methanol (5 mL). The mother liquor gave pure methyl octadeca-9*E*,11*Z*-dienoate (**9**, 0.2 g, 15%). IR (cm^{-1}) 2930, 2856, 1742, and 945; UV_{max} 232; ^1H NMR (δ_H) 0.88 (*t*, *J* = 6 Hz, 3H, CH_3), 1.20–1.50 (*m*, 18H, CH_2), 2.00–2.10 (*m*, 4H, 8-H_2, 13-H_2), 2.30 (*t*, *J* = 7 Hz, 2H, 2-H_2), 3.66 (*s*, 3H, $COOCH_3$), 5.30 (*m*, 1H, 12-*H*), 5.66 (*t*, *J* = 5.4 Hz, 1H, 11-*H*), 5.93 (*m*, 1H, 9-*H*), 6.24 (*t*, *J* = 12.9 Hz, 1H, 10-*H*).

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Growth Inhibitory Effects of Liposome-Associated 1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine

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ABSTRACT: The growth inhibitory effects of 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH_3) and various liposome compositions of ET-18-OCH_3 were compared in a standardized growth inhibition assay utilizing a diverse tumor cell line panel including cell lines expressing multidrug resistance. ET-18-OCH_3 and ELL-12 (4:3:1:2, dioleoylphosphatidylcholine/cholesterol/dioleoylphosphatidylethanolamine–glutaric acid/ET-18-OCH_3), an optimal liposomal ET-18-OCH_3 formulation, inhibited growth in the micromolar range in drug-sensitive and -resistant cells. In general, ET-18-OCH_3–liposomes were about twofold less growth inhibitory than ET-18-OCH_3. However, the known hemolytic effects of ET-18-OCH_3 were greatly reduced, up to 20 or more times, by liposome association. The effects of ET-18-OCH_3 and ELL-12 were compared in intracellular [Ca^{2+}] modulation and DNA fragmentation assays. ET-18-OCH_3 elicited both concentration- and serum-dependent transient and permanent increases in intracellular [Ca^{2+}]. In contrast, ELL-12 did not modulate intracellular [Ca^{2+}]. ET-18-OCH_3 and ELL-12 similarly affected DNA fragmentation, which may be indicative of apoptosis. The results suggest that, although the specific growth inhibitory effects of ET-18-OCH_3 and ELL-12 are similar, associating ET-18-OCH_3 with stable well-characterized liposomes eliminates nonspecific cell membrane-associated lytic effects.

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Alkylphosphocholines, such as the ether lipid 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH_3), are antitumor agents that differ from many other cancer therapeutic agents in that they do not interact directly with DNA and are not myelosuppressive in animal models (1,2).

Alkylphosphocholines, including ET-18-OCH_3, have been reported to preferentially accumulate in the plasma membranes of transformed cell lines (3,4) and may modulate membrane-associated signal transduction pathways (5,6). For example, ET-18-OCH_3 directly inhibits two important enzymes involved in intracellular signal transduction, phospholipase C and protein kinase C (7). Furthermore, ET-18-OCH_3 was shown to inhibit transient increases in intracellular Ca^{2+} levels associated with receptor-mediated growth factor and mitogen binding, perhaps by modulating the phosphatidylinositol turnover pathway (8–10). ET-18-OCH_3 has also elicited acute transient intracellular [Ca^{2+}] increases which may be related to inositol triphosphate-induced [Ca^{2+}] transients (11,12). Recent evidence (13) suggests that ET-18-OCH_3 may modulate transcription by inhibiting a nuclear factor kappa B pathway by a protein kinase C-dependent mechanism. ET-18-OCH_3 has also been reported to interfere with cell cycle progression in transformed cells. For example, ET-18-OCH_3 caused an accumulation of BAC1.2F5 cells, a macrophage-like cell line, in G2/M phase (14) and induced DNA fragmentation and morphological changes, indicative of programmed cell death, in both BAC1.2F5 and HL-60, a human leukemia cell line (14,15). These studies suggest that the anticancer effects of ET-18-OCH_3 are mediated by modulation of intracellular signal transduction and/or cell cycle progression.

Although ET-18-OCH_3 has demonstrated antitumor activity in various animal models, only modest clinical activity has been demonstrated, and it has significant clinical toxicities, including hemolysis (1,16). Since the association of several drugs with liposomes has been shown to attenuate nonspecific toxicity *in vivo* (17–19), we thought that association of ET-18-OCH_3 with liposomes would reduce its toxicity and perhaps enhance its delivery to tumors. We have described previously the biophysical characterization of a number of ET-18-OCH_3 liposome formulations (20,21). In this report the growth inhibitory properties, effects on intracellular Ca^{2+} mobilization, and the effects on DNA fragmentation of some of these formulations were compared to those of free ET-18-OCH_3.

MATERIALS AND METHODS

Cell lines. A549, human nonsmall cell lung carcinoma; MCF 7, human breast carcinoma; MCF 7/adr, multidrug-resistant human breast carcinoma; Ovcar 3, human ovarian carcinoma;

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Abbreviations: CH, cholesterol; ELL-12, dioleoylphosphatidylcholine/cholesterol/dioleoylphosphatidylethanolamine–glutaric acid, 4:3:1:2; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; GI_{50}, drug concentration which inhibits cell growth 50%; HI_5, drug concentration required to cause 5% hemolysis; PBS, phosphate-buffered saline without Ca^{2+} and Mg^{2+}; RFU, relative fluorescence units; SRB, sulforhodamine B; TI, therapeutic index.

P388, murine leukemia; and P388/adr, drug-resistant murine leukemia, were obtained from the DCT Tumor Repository (NCI–Frederick Cancer Research Facility, Frederick, MD). Du145, human prostate carcinoma, was obtained from the American Type Culture Collection (Rockville, MD). L1210 and L1210/vmdr, murine leukemias, were a generous gift from Dr. Alan Sartorelli (Yale University School of Medicine, New Haven, CT). The L1210/vmdr subline was L1210 transfected with a vector (pHaMDR1/A) containing the *mdr 1* gene.

ET-18-OCH_3 and ET-18-OCH_3 liposome formulations. The L-isomer of ET-18-OCH_3 was purchased from Calbiochem (La Jolla, CA) or Avanti Polar Lipids, Inc. (Alabaster, AL). The other lipids used for the liposome formulations were all purchased from Avanti Polar Lipids, Inc. ET-18-OCH_3 liposome formulations, listed in Table 1, were prepared using the solvent evaporation method (22). Briefly, ET-18-OCH_3 and other lipids were dissolved in chloroform/methanol (2:1, vol/vol) and mixed in the desired molar lipid ratios of neutral phospholipid/cholesterol/ET-18-OCH_3/glutaric acid-derivatized phospholipid. After thorough mixing, the organic solvent was vacuum-evaporated at 45°C, and the thin dried film was hydrated with Dulbecco's phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} (Mediatech, Herndon, VA) for 1 h at room temperature or above the transition temperature for the lipids. The resulting multilamellar preparations were extruded 10 times through 100-nm double-stacked Nucleopore filters using an extruder device (Lipex Biomembranes, Vancouver, British Columbia, Canada). The liposomes were examined by light microscopy to check morphology, and size was determined using a Nicomp Model 370 Submicron Particle Sizer system (Santa Barbara, CA). Liposome size ranged from 96–126 nm. ET-18-OCH_3 and other lipid concentrations in the final preparations were determined using reverse-phase high-performance liquid chromatography using an evaporative light-scattering detector with 4% water in methanol as the mobile phase.

In vitro *growth inhibition.* A sulforhodamine B (SRB) assay was used to determine relative *in vitro* drug sensitivity using the GI_{50} parameter (the concentration of drug which inhibits cell growth 50%). The detailed methodology of the SRB assay is described elsewhere (23,24). Briefly, 96-well microtiter plates were inoculated with 100 µL of the above cell lines at optimal cell densities, 3500–15,000 cells/well, in medium RPMI 1640 with L-glutamine (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY) for all cell lines except for Du145 which was cultured in Eagle's minimal essential medium with Earle's salts and L-glutamine (Mediatech) supplemented with 10% fetal bovine serum (Life Technologies). The plates were then incubated for 24 h at 37°C, 100% humidity, and 5% CO_2. Medium (100 µL) was added to designated "time zero" plates which were fixed with 50 µL of 50% trichloroacetic acid (wt/vol) (Sigma, St. Louis, MO) (adherent cells) or 80% trichloroacetic acid (suspension cells) and refrigerated at 4°C for 1 h. The supernatant was then discarded and the plates were rinsed six times with water and air-dried. ET-18-OCH_3 or a liposome formulation of ET-18-OCH_3 was added (100-µL aliquots) to the nonfixed plates at twice the predetermined highest concentration and serially diluted across the plates. Growth control wells received 100 µL of medium. There were triplicate wells on each plate and duplicate plates (six wells) for each drug concentration. The cells were then incubated for 72 h under the above conditions. The treated plates were acid-fixed, rinsed, and dried as above. One hundred microliters of 0.4% SRB (Sigma) in 1% acetic acid (Sigma) was added to the plates and incubated at room temperature for 10 min. Unbound stain was removed by rinsing the plates three times with 1% acetic acid. The plates were air-dried for 24 h. The bound stain was solubilized with 100 µL of 10 mM Tris buffer (Sigma), and the optical density was read spectrophotometrically at 490 nm (Microplate Reader Model 3550-UV; Biorad, Hercules, CA). The raw optical density data were imported into an Excel (Microsoft, Redmond, WA) spreadsheet to determine dose responses. Percentage growth was calculated as follows: $(T - T_0)/(C - T_0) \times 100$ where T = mean optical density of treated wells at a given drug concentration, T_0 = mean optical density of time zero wells, and C = mean optical density of control wells. If $T < T_0$, which indicates that cell death has occurred, then percentage cell death was calculated as $(T - T_0)/(T_0) \times 100$. By varying drug concentration, dose-response curves were generated, and the GI_{50} values were calculated. The GI_{50} values for each experiment were calculated using data obtained from the six wells as described above. The mean GI_{50} values and standard deviations were calculated from the GI_{50} data from each independent experiment. Data obtained using the growth inhibition assay are shown in Tables 1–3 and Figures 1 and 3.

Cell survival assay. Cell survival was determined using a colony-forming assay modified from Fugiwara *et al.* (25). Briefly, T75 culture flasks (Corning Costar, Boston, MA), one for each drug concentration and a growth control, were inoculated with 7.5×10^5 MCF 7 cells (adherent culture) or L1210 cells (suspension culture) in medium (RPMI 1640 with L-glutamine supplemented with 10% fetal bovine serum), and incubated at 37°C, 5% CO_2, and 100% humidity for 24 h (MCF 7) or 6 h (L1210). The supernatant was discarded, and fresh medium containing various concentrations of ET-18-OCH_3 was added to the specified flasks which were incubated at the above conditions for 72 h. In the case of MCF 7, the supernatant from each flask was removed and the cells were rinsed with PBS, and trypsinized using 0.05% trypsin-EDTA (Life Technologies) to dissociate the cell monolayer. The cells were resuspended in the above supernatant so as to include nonadherent (dead or dying) cells in the subsequent cell count. The cell suspension was centrifuged at $180 \times g$, and the cell pellet was resuspended in fresh medium. In the case of L1210, the cell suspension was centrifuged at $180 \times g$, and the cell pellet was resuspended in fresh medium. The cells were counted (Coulter Counter ZM; Coulter Electronics, Inc., Hialeah, FL) and plated in 100-mm plastic petri dishes (500, 200, or 100 cells/plate) (MCF 7) or T75 flasks at 500 cells/flask (L1210).

The petri dishes (or flasks) were incubated 7 to 12 d at the above conditions. In the case of MCF 7, the surviving fraction was determined relative to the mean colony-forming efficiency (number of colonies/the number of cells plated) of the control plates at the different cell densities. The surviving fraction was calculated by dividing the mean colony-forming efficiency of the cells exposed to ET-18-OCH_3 by the control colony-forming efficiency. L1210 survival was determined from number of treated cells as a percentage of controls. The mean surviving cell fraction for each drug concentration was calculated from five duplicate plates or flasks in each experiment. The mean surviving fractions from three individual experiments were averaged and standard deviations were calculated. Data from experiments using the cell survival assay are shown in Figure 2.

Hemolysis assay. Hemolysis was quantitated by measuring hemoglobin release into an isotonic buffer after the addition of ET-18-OCH_3 or liposomal ET-18-OCH_3. Varying concentrations of ET-18-OCH_3 or liposomal ET-18-OCH_3 were incubated with 4% human red blood cell (washed in PBS three times) suspensions in PBS at 37°C for 20 h. The percentage lysis was calculated using lysis after the addition of water as a positive control (100% lysis) and lysis after the addition of PBS as the negative control. After incubation, the samples were centrifuged at 2000 × *g* for 10 min, and the absorbance of the supernatant was measured at 550 nm. Absorbance correlates with hemolysis ($r^2 > 0.9$). ET-18-OCH_3 and the different liposomal formulations were evaluated based on the calculated HI_5 value (the ET-18-OCH_3 concentration required to cause 5% hemolysis). Each formulation was tested in triplicate in each experiment.

Variable drug exposure times (pulse–chase). A549 and MCF 7 cells were used in a modification of the SRB growth inhibition assay to evaluate the effects of ET-18-OCH_3 and liposomal ET-18-OCH_3 at different exposure times. The cell lines were inoculated at 5000 cells/well and incubated for 24 h, as above. "Time zero" plates were fixed, and ET-18-OCH_3 or liposomal ET-18-OCH_3 was added to the rest of the plates. The cells were incubated with the drugs for five different exposure times (1, 4, 8, 24, and 72 h), two plates per exposure time. The posttreatment incubation time totaled 72 h; therefore, the cells exposed for 1, 4, 8, or 24 h were washed twice with fresh medium and incubated 71, 68, 64, or 48 h, respectively. The 72-h time was a continuous exposure. After the posttreatment exposure time, the cells were processed as described for the *in vitro* growth inhibition assay.

Intracellular Ca^{2+} determination. Modulation of intracellular Ca^{2+} was determined utilizing the Ca^{2+} marker Fluo3 (Molecular Probes, Eugene, OR). In order to "load" the Ca^{2+} marker in the cells, the cell-permeable, acetoxy methyl ester derivative of Fluo3, Fluo3AM, was dissolved in a nonionic detergent, 20% Pluronic F-127 (Molecular Probes) in dimethylsulfoxide (J.T. Baker, Phillipsburg, NJ) at 6 mM, then diluted to 6 μM in PBS. The Fluo3AM solution was added to a suspension of L1210 cells. The final concentration of Fluo3AM was 3 μM, and the cell density was either 6×10^5 or 9×10^5 cells/mL. Fluo3AM was incubated with the cells for 30 min at 37°C. Following the 30-min incubation, the cells were centrifuged at 180 × *g*, rinsed with cold PBS, centrifuged, and resuspended in either warm PBS or 10% fetal bovine serum in PBS (pH adjusted to 7.2). The cell suspension was aliquoted into a cuvette, and the relative fluorescence was monitored continuously after the addition of increasing concentrations of ET-18-OCH_3 or a liposomal formulation of ET-18-OCH_3. The Ca^{2+}-associated fluorescence was measured utilizing a photon-counting spectrofluorometer (Photon Technology International, Inc., South Brunswick, NJ) configured for a time-based measurement with the excitation and emission wavelengths set for 470 and 530 nm, respectively (the bandwidth slits were set at 4 nm). Digitonin (Sigma), a nonionic detergent, was added to determine the fluorescence maximum. The fluorescence minimum was calculated at the basal Ca^{2+}-associated fluorescence level when percentage relative fluorescence measurements were compared. EDTA (Sigma) was added to quench fluorescence (fluorescence minimum for $[Ca^{2+}]$ calculation). The $[Ca^{2+}]$ was determined using a method described by Grynkiewicz *et al.* (26). A standard curve was obtained using a calcium calibration buffer kit (Molecular Probes), and from the standard curve, unknown Ca^{2+} concentrations were calculated as: $[Ca^{2+}] = \log_{10}[(F - F_{min})/(F_{max} - F)]$ where F = relative fluorescence units (RFU) of unknown, F_{max} = RFU of sample after the addition of digitonin (50 μM), and F_{min} = RFU of sample after the addition of 50 μM EDTA. In order to differentiate between Ca^{2+} being liberated from intracellular stores or extracellular Ca^{2+}, L1210 cells were assayed with or without the addition of EDTA in the suspension buffer. Ca^{2+} originating from intracellular stores was determined by chelating extracellular Ca^{2+} with EDTA. Total Ca^{2+} originating from both intracellular and extracellular sources was determined without the addition of EDTA.

DNA fragmentation analysis. A modification of the DNA fragmentation analysis described by Gauthier *et al.* (27) was used. L1210 control cells (1×10^6) or cells treated with ET-18-OCH_3 or liposomal ET-18-OCH_3 were washed with PBS and resuspended in 20 μL of lysis buffer [50 mM Tris HCl, pH 8.0, 10 mM EDTA, 0.5% Na laurylsarcosinate containing 1.25 mg/mL proteinase K (Gibco, Grand Island, NY)] and incubated at 50°C for 1 h. Then 10 μL (10.0 mg/mL) of RNase A (Boehringer Mannheim, Indianapolis, IN) was added and incubated at 50°C for 1 h. The reaction was stopped by the addition of 10 μL of loading buffer [10 mM EDTA, pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll 400]. The samples were heated at 70°C for 10 min, 10 μL of melted 2% agarose was added, and the whole samples were loaded onto 2% agarose gels. The samples and a marker [100 base pair DNA ladder (Gibco)] were electrophoresed at 50 V for 5–6 h in Tris-borate-EDTA buffer [100 mM Tris, 83 mM boric acid, 1 mM EDTA (pH 8.3)] on a 2% agarose gel. Gels were then stained with 0.5 μg/mL ethidium bromide.

RESULTS

In vitro *growth inhibition by ET-18-OCH$_3$.* A representative dose-response curve for MCF 7 cells treated with ET-18-OCH$_3$ using the SRB assay is shown in Figure 1. There was a twofold difference between the GI_{50} and total growth inhibition (GI_{100}) and a greater than fivefold difference between the GI_{50} and the extrapolated value for 100% cell death (LC_{100}). There were differences in drug sensitivity between cell types. The GI_{50} values for free ET-18-OCH$_3$ for six cell lines investigated were: A549, 6.5 μM; MCF 7, 16.8 μM (Table 1); L1210, 3.9 μM; P388, 5.0 μM (Table 2); Ovcar 3, 6.2 μM; and Du145, 14.0 μM (separate experiments). It is of interest to note that prolonged incubation of MCF 7 cells with ET-18-OCH$_3$ did not select for cells resistant to ET-18-OCH$_3$ or other chemotherapeutic agents. This was shown in experiments where the growth inhibitory effects of ET-18-OCH$_3$, doxorubicin, or 5-fluorouracil were determined against MCF 7 cells before and after the cells were treated for 72 h with 60 μM ET-18-OCH$_3$. The GI_{50} values were 15.2, 0.018, and 4.9 μM before and 13.1, 0.013, and 4.5 μM after treatment with ET-18-OCH$_3$, doxorubicin, and 5-fluorouracil, respectively.

To further characterize the growth inhibitory effects of ET-18-OCH$_3$ on MCF 7 and L1210, we evaluated cells in a cell survival assay (Fig. 2). MCF 7 and L1210 were chosen for this analysis because there was a relatively large (fourfold) difference between their GI_{50} values as described above. Figure 2 shows that greater than 30% MCF 7 cells survived a 72-h treatment with 60 μM ET-18-OCH$_3$ and most cells survived 72 h at the GI_{50} (16.8 μM). In contrast, L1210 cells were much more sensitive to the cytotoxic effects of ET-18-OCH$_3$. Few cells survived at the GI_{50} concentration (3.9 μM), and the surviving fraction was reduced by at least 100-fold at 5 μM. The results with L1210 cells are similar to those reported for BG-1 human ovarian carcinoma cells (25). It can also be seen in Figure 2 that when MCF 7 cells were treated with 7.5 μM ET-18-OCH$_3$ there was a slight increase in the number of colonies ($P < 0.05$, *t*-test) compared to controls, which suggests that at low concentrations ET-18-OCH$_3$ may act as a mitogen in this cell line. Similar results were reported by Lohmeyer and Workman (28) using HT 29 cells. Clearly, however, ET-18-OCH$_3$ functions primarily as a cytostatic rather than a cytotoxic agent against MCF 7 cells at concentrations in the GI_{50} range, whereas the opposite is true for L1210 cells.

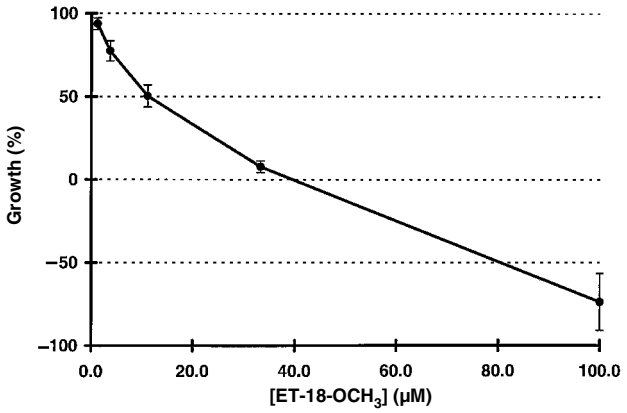

FIG. 1. Dose-response curve of MCF 7 cells after a 72-h exposure at increasing 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH$_3$) concentrations. The curve was generated using the sulforhodamine B assay described in the Materials and Methods section. The means and standard deviations were calculated from three independent experiments.

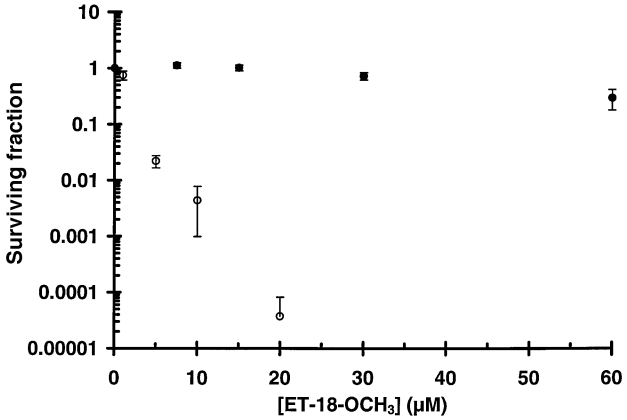

FIG. 2. Survival of MCF 7 (●) and L1210 (○) cells following a 72-h exposure to increasing ET-18-OCH$_3$ concentrations. After drug exposure the cells were washed, plated, and incubated for 7–12 d. Then the number of MCF 7 colonies or L1210 cells was determined as a percentage of the control (untreated) cell or colony number at each drug concentration. The mean fractional cell survival was calculated from three independent experiments. The error bars represent the standard deviations. For abbreviation see Figure 1.

In vitro *growth inhibition and hemolysis by ET-18-OCH$_3$ liposomes.* We have previously shown that ET-18-OCH$_3$ can be stably associated with liposomes, and the stability of the association is composition-dependent, as measured by several parameters, including human red cell lysis (20,21). Table 1 compares the growth inhibitory and hemolytic effects of a number of ET-18-OCH$_3$ liposome compositions. In general, the various ET-18-OCH$_3$ liposomes were 1.5- to 3.0-fold less growth inhibitory than ET-18-OCH$_3$. Growth inhibition was not strongly dependent on liposome composition. Hemolysis by the different formulations was compared using the calculated HI_5 value. As reported previously (20,21), all liposome formulations were less hemolytic than free ET-18-OCH$_3$ (HI_5 = 5.6 μM). For the egg phosphatidylcholine/cholesterol(CH)/dipalmitoylphosphatidylethanolamine–glutaric acid/ET-18-OCH$_3$, egg phosphatidylcholine/CH/1-palmitoyl-2-oleoylphosphatidylethanolamine–glutaric acid/ET-18-OCH$_3$, and distearoylphosphatidylcholine/CH/dipalmitoylphosphatidylethanolamine–glutaric acid/ET-18-OCH$_3$ series of formulations, the higher the mole ratio of ET-18-OCH$_3$, the more hemolytic the formulation. Dioleoylphosphatidylcholine/CH/dioleoylphosphatidylethanolamine–glutaric acid/ET-18-OCH$_3$, 4:3:1:2 (ELL-12) and dioleoylphosphatidylethanolamine/CH/dioleoylphosphatidylethanol-

TABLE 1
GI_{50}[a] Values of A549 and MCF 7 Cells Treated with ET-18-OCH_3 or ET-18-OCH_3 Liposomes and the HI_5[b] Values Associated with ET-18-OCH_3 and ET-18-OCH_3 Liposomes[c]

ET-18-OCH_3 liposome formulation	A549 GI_{50} ± SD (μM)	MCF 7 GI_{50} ± SD (μM)	Hemolysis HI_5 ± SD (μM)
Free ET-18-OCH_3	6.5 ± 1.9 (6)	16.8 ± 6.3 (9)	5.6 ± 0.9 (3)
EPC/CH/DPPE-GA/ET-18-OCH_3			
5:3:1:1	<7.5	n.d.	33
4:3:1:2	9.2 ± 3.4 (3)	24.7 ± 11.9 (3)	19
3:3:1:3	<7.5	n.d.	14.1 (2)
1:3:1:5	<7.5	n.d.	9
EPC/CH/POPE-GA/ET-18-OCH_3			
5:3:1:1	18.3 (2)	>61.7 (2)	31
4:3:1:2	11.1 (2)	29.7 (2)	31.5 (2)
3:3:1:3	11.7 (2)	28.0 (2)	20
1:3:1:5	9.9 (2)	17.9 (2)	14
DSPC/CH/DPPE-GA/ET-18-OCH_3			
5:3:1:1	<7.5	n.d.	20
4:3:1:2	<10.6	33.9	18
3:3:1:3	<7.5	n.d.	11
1:3:1:5	<7.5	n.d.	9
EPC/CH/DOPE-GA/ET-18-OCH_3			
4:3:1:2	15.8	36.0	140
DOPC/CH/DOPE-GA/ET-18-OCH_3			
4:3:1:2 (**ELL-12**)	13.7 ± 1.3 (3)	28.8 ± 11.4 (4)	250 ± 25 (5)
DOPE/CH/DOPE-GA/ET-18-OCH_3			
4:3:1:2 (**ELL-20**)	n.d.	35.3	640
CH/DPPG/ET-18-OCH_3, 5:3:2	n.d.	>75	72
CH/DOPE-GA/ET-18-OCH_3, 5:1:4	n.d.	40.5	166
DOPC/DOPE-GA/ET-18-OCH_3, 7:1:2	n.d.	22.5	87
DOPE/DOPE-GA/ET-18-OCH_3, 7:1:2	n.d.	33.8	108
POPE/DOPE-GA/ET-18-OCH_3, 4:2:4	n.d.	24.8	65

[a]GI_{50}, the drug concentration that inhibits cell growth 50% after 72-h drug treatment (sulforhodamine B assay).
[b]HI_5, the drug concentration required to cause 5% hemolysis.
[c]If there were three or more experiments, a mean and standard deviation were calculated and the number of experiments is listed in parentheses. If there were fewer than three experiments, the individual GI_{50} values were averaged. If a GI_{50} value is preceded by a > or < symbol, the GI_{50} value fell outside the dose range of that specific experiment and could not be calculated. Abbreviations: n.d., not done; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; EPC, egg phosphatidylcholine; CH, cholesterol; DPPE-GA, dipalmitoylphosphatidylethanolamine–glutaric acid; POPE-GA, 1-palmitoyl-2-oleoylphosphatidylethanolamine–glutaric acid; DSPC, distearoylphosphatidylcholine; DOPE-GA, dioleoylphosphatidylethanolamine–glutaric acid; DOPC, dioleoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol.

amine–glutaric acid/ET-18-OCH_3, 4:3:1:2 (ELL-20) were clearly the least hemolytic formulations.

Therapeutic index (TI). An important *in vivo* toxicity of ET-18-OCH_3 is hemolysis (1,29). In order for ET-18-OCH_3 or ET-18-OCH_3-liposomes to be an effective growth inhibitory, anticancer agent, growth inhibition must result at concentrations lower than those that cause hemolysis. A theoretical TI was calculated by dividing the HI_5 by the GI_{50} determined for A549 and MCF 7 cells. Table 3 shows the TI for selected ET-18-OCH_3-liposome preparations. A TI less than one indicates hemolysis should occur at lower concentrations than those required to inhibit tumor cell growth. A TI greater than one suggests cell growth will be inhibited without measurable hemolysis. As Table 3 shows, free ET-18-OCH_3 has a TI less than one and, therefore, growth inhibitory effects would not occur without hemolysis. TI calculated for the liposome preparations were all found to be greater than one. ELL-12 and ELL-20 had a TI greater than eight, suggesting these formulations could achieve growth-inhibitory concentrations without hemolysis. Ahmad *et al.* (30) have shown that ELL-12 is less toxic and has enhanced therapeutic effects compared to ELL-20 *in vivo*, so in the remainder of the experiments described in this report ELL-12 was used as the liposome formulation for comparison with ET-18-OCH_3.

ET-18-OCH_3, ELL-12, and drug resistance. Table 2 illustrates that free ET-18-OCH_3 and ELL-12 were growth inhibitory against several drug-resistant cell lines characterized by P-glycoprotein expression. Multidrug-resistant cell lines were only 1.5–3.0-fold more resistant to free or liposomal ET-18-OCH_3 compared to the parental cell lines. However, the cell lines expressing P-glycoprotein were 100- to 1000-fold more resistant to agents such as doxorubicin, paclitaxel, or colchicine.

TABLE 2
GI_{50} Values of Parent and Resistant Cell Line Pairs Treated with ET-18-OCH_3, ET-18-OCH_3 Liposomes, or Selected Standard Agents

Agent	MCF 7[a] GI_{50} ± SD (μM)	MCF 7/adr[a] GI_{50} ± SD (μM)	Relative resistance[b]
Free ET-18-OCH_3	16.8 ± 6.3 (8)	29.6 ± 4.0 (6)	2
DOPC/CH/DOPE-GA/ET-18-OCH_3, 4:3:1:2[c]	28.8 ± 11.4 (4)	74.0 ± 35.7	3
EPC/CH/POPE-GA/ET-18-OCH_3, 4:3:1:2	29.7 (2)	45.7 (2)	2
DSPC/CH/DPPE-GA/ET-18-OCH_3, 4:3:1:2	33.9	105.5	3
EPC/CH/DOPE-GA/ET-18-OCH_3, 4:3:1:2	36.0	>68.6	>2
EPC/CH/DPPE-GA/ET-18-OCH_3, 4:3:1:2	24.7 ± 11.9 (3)	55.6	2
Doxorubicin	0.022 ± 0.014 (11)	11.29 ± 3.39 (6)	500
Paclitaxel	0.0022 ± 0.0003 (3)	3.2700 ± 0.3588 (3)	1500
Colchicine	0.0048 (2)	2.3975 (2)	500

Agent	P388 GI_{50} ± SD (μM)	P388/adr GI_{50} ± SD (μM)	Relative resistance[b]
Free ET-18-OCH_3	5.0 ± 1.1 (5)	6.9 ± 2.3 (5)	1.4
DOPC/CH/DOPE-GA/ET-18-OCH_3, 4:3:1:2[c]	7.2 ± 2.7 (3)	12.4 ± 1.5 (3)	2
EPC/CH/POPE-GA/ET-18-OCH_3, 4:3:1:2	5.1 (2)	9.3 (2)	2
DSPC/CH/DPPE-GA/ET-18-OCH_3, 4:3:1:2	10.5	20.3	2
EPC/CH/DOPE-GA/ET-18-OCH_3, 4:3:1:2	7.5	13.3	2
EPC/CH/DPPE-GA/ET-18-OCH_3, 4:3:1:2	5.1 (2)	8.2 (2)	2
Doxorubicin	0.014 ± 0.010 (3)	4.343 ± 1.814 (3)	300
Paclitaxel	0.0215 (2)	2.6117 ± 0.9368 (3)	100
Colchicine	0.0222 ± 0.0058 (3)	3.1800 ± 0.9924 (3)	100

Agent	L1210 GI_{50} ± SD (μM)	L1210/vmdr GI_{50} ± SD (μM)	Relative resistance[b]
Free ET-18-OCH_3	3.9 ± 1.2 (3)	10.3 (2)	3
DOPC/CH/DOPE-GA/ET-18-OCH_3, 4:3:1:2[c]	4.4 ± 1.3 (3)	11.5 (2)	3
EPC/CH/POPE-GA/ET-18-OCH_3, 4:3:1:2	n.d.	n.d.	n.d.
DSPC/CH/DPPE-GA/ET-18-OCH_3, 4:3:1:2	n.d.	n.d.	n.d.
EPC/CH/DOPE-GA/ET-18-OCH_3, 4:3:1:2	n.d.	n.d.	n.d.
EPC/CH/DPPE-GA/ET-18-OCH_3, 4:3:1:2	n.d.	n.d.	n.d.
Doxorubicin	0.0421 ± 0.0191 (4)	0.987 (2)	20
Paclitaxel	0.0288 ± 0.0159 (3)	>3.3590 (3)	>100
Colchicine	0.0228 (2)	2.6642 ± 1.5086 (3)	100

[a]See corresponding footnote in Table 1. For abbreviations see Table 1.
[b]The relative resistance was determined by: GI_{50} (resistant cell line)/GI_{50} (parent cell line).
[c]DOPC/CH/DOPE-GA/ET-18-OCH_3, 4:3:1:2 = ELL-12.

Variable drug exposure times (pulse-chase). In all the previous growth inhibition experiments, the effects of ET-18-OCH_3 and liposomal ET-18-OCH_3 were determined under continuous drug exposure conditions. Figure 3 shows the dose-response curves of MCF 7 cells when exposed to variable ET-18-OCH_3 or ELL-12 concentrations for increasing periods of time. The effects of ET-18-OCH_3 were both time- and concentration-dependent. An ET-18-OCH_3 concentration of 150 μM completely inhibited cell growth after a 1-h exposure and was cytotoxic at longer exposure times. When cells were exposed to 16.7 μM and 5.6 μM ET-18-OCH_3, growth inhibition was not apparent until after 24 h of drug exposure. The effects of ELL-12 were again both time- and concentration-dependent, but the dose-response curves were not as steep for ET-18-OCH_3.

TABLE 3
Theoretical Therapeutic Indices (TI) Based on HI_5 and GI_{50} Values[a]

ET-18-OCH_3 liposome formulation	A549 TI	MCF 7 TI
Free ET-18-OCH_3	0.86	0.33
EPC/CH/POPE-GA/ET-18-OCH_3, 4:3:1:2	2.84	1.06
EPC/CH/DOPE-GA/ET-18-OCH_3, 4:3:1:2	8.86	3.89
DOPC/CH/DOPE-GA/ET-18-OCH_3, 4:3:1:2 (**ELL-12**)	18.25	8.68
DOPE/CH/DOPE-GA/ET-18-OCH_3, 4:3:1:2 (**ELL-20**)	n.d.	18.13

[a]TI is HI_5/GI_{50}. If TI <1, then the GI_{50} cannot be reached without causing hemolysis (HI_5 >5%). When TI >1, there is a "window" for inhibiting tumor cell growth without causing hemolysis (HI_5 <5%). See Table 1 for abbreviations.

Effects of ET-18-OCH_3 or ELL-12 on intracellular Ca^{2+}. The purpose of the Ca^{2+} measurements was to compare the modulation of intracellular [Ca^{2+}] by free ET-18-OCH_3 with that of ELL-12. The important role Ca^{2+} plays in signal transduction is well known, and agents which may perturb signal transduction pathways may be expected to have effects on intracellular Ca^{2+}, as discussed in References 5–7. When the cell plasma membrane is severely damaged, the levels of in-

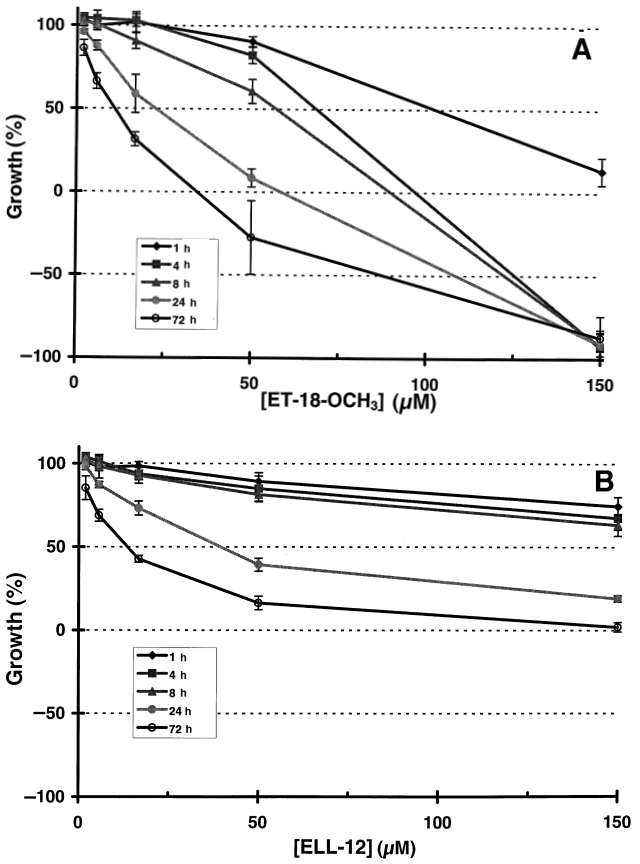

FIG. 3. The effects of free (A) ET-18-OCH_3 or (B) ELL-12 (4:3:1:2 dioleoylphosphatidylcholine/cholesterol/dioleoylphosphatidylethanolamine–glutaric acid/ET-18-OCH_3) on MCF 7 cells treated with increasing drug concentrations for specified periods. After drug exposure, the cells were washed and re-incubated. After a total of 72-h incubation the cells were processed as described in the Materials and Methods section, using the sulforhodamine B assay. Cells were not washed or reincubated for the continuous 72-h exposure. For abbreviation see Figure 1.

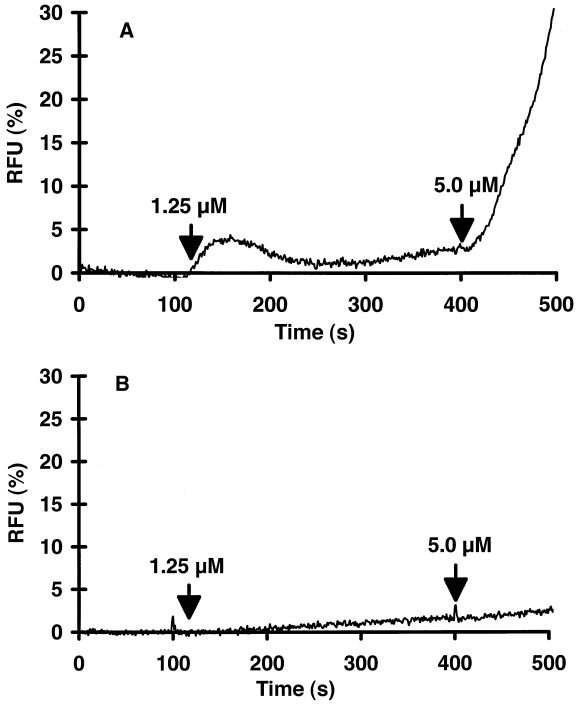

FIG. 4. The effects of ET-18-OCH_3 or ELL-12 on intracellular [Ca^{2+}] in L1210 cells incubated in phosphate-buffered saline without Ca^{2+} and Mg^{2+}. L1210 cells were loaded with the Ca^{2+} marker Fluo3 (Molecular Probes, Eugene, OR) and treated with increasing concentrations of ET-18-OCH_3 or ELL-12. (A) Represents the time-based trace of ET-18-OCH_3, and (B) represents the ELL-12 trace. At 100 s, 1.25 μM ET-18-OCH_3 or ELL-12 (final concentration) was added to the cells. At 400 s, 5 μM ET-18-OCH_3 or ELL-12 (final concentration) was added to the cells. RFU = relative fluorescence units. For other abbreviations see Figures 1 and 3.

tracellular ions, including Ca^{2+}, will change dramatically. However, when plasma membrane damage is sublethal, changes in [Ca^{2+}] are more subtle and may involve release of Ca^{2+} from intracellular sites as well as influx of extracellular Ca^{2+}. We were interested in these changes, as we already had shown (Table 1) that association of ET-18-OCH_3 with liposomes such as ELL-12 abrogated the lytic effects. Figure 4 shows the effects of ET-18-OCH_3 or ELL-12 on intracellular [Ca^{2+}] in L1210 cells suspended in PBS. (L1210 cells were used in these experiments because of their sensitivity to ET-18-OCH_3.) The addition of 1.25 μM free ET-18-OCH_3 caused a transient increase in intracellular [Ca^{2+}] (Fig. 4A), resulting from both intracellular Ca^{2+} liberation from intracellular stores and extracellular [Ca^{2+}] diffusion into the cytoplasm. The total change in intracellular [Ca^{++}], originating from both intracellular and extracellular sources, was 13.0 ± 1.1 nM, whereas the change in intracellular [Ca^{2+}], originating from intracellular sources, was 6.7 ± 3.0 nM. The addition of 5 μM free ET-18-OCH_3 elicited a large permanent increase in intracellular [Ca^{2+}], most likely owing to an influx of external Ca^{2+}. The large increase in [Ca^{2+}] was associated with a substantial decrease in cell viability, approximately 90%, as indicated by trypan blue exclusion. In contrast, the addition of 1.25 μM ET-18-OCH_3 resulted in a less than 10% loss in viability. ELL-12 did not affect intracellular [Ca^{2+}] at either concentration (1.25 or 5 μM) (Fig. 4B). The slow increase observed in percentage RFU was consistent with the increase seen in the negative controls. Even though Fluo3 is reported to be cell impermeable, it appears to leak slowly under these conditions.

Intracellular [Ca^{2+}] was also monitored in the presence of 10% fetal bovine serum which more closely compares with cell growth conditions in the growth inhibition assays. Figure 5A indicates that 5 μM ET-18-OCH_3 or higher was needed in serum to elicit intracellular [Ca^{2+}] transients similar to the transients seen after the addition of 1.25 μM ET-18-OCH_3 in PBS. In contrast, ELL-12 did not elicit measurable [Ca^{2+}] transients in serum, even at the highest concentrations tested (Fig. 5B). Sequential addition of increasing concentrations of ET-18-OCH_3 did not appear to inhibit the modulation of [Ca^{2+}]. The slow increase in fluorescence observed was similar to that in controls. Fluo3 appears to leak more rapidly in 10% serum than in PBS. It is noteworthy that under conditions where both ET-18-OCH_3 and ELL-12 were growth-in-

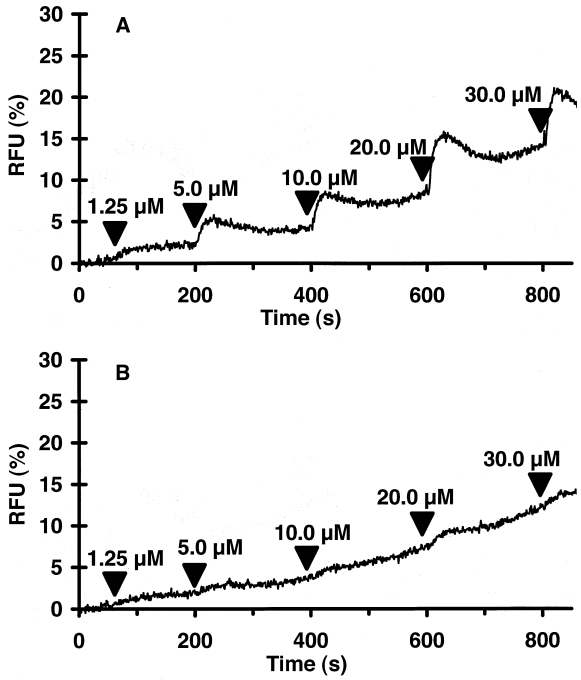

FIG. 5. The effects of ET-18-OCH_3 or ELL-12 on intracellular [Ca^{2+}] in L1210 cells incubated in 10% fetal bovine serum. L1210 cells were loaded with the Ca^{2+} marker Fluo3 and treated with increasing concentrations of ET-18-OCH_3 or ELL-12. (A) Represents the time-based trace of ET-18-OCH_3, and (B) represents the ELL-12 trace. Increasing concentrations of ET-18-OCH_3 or ELL-12 (reported as final concentrations) were added to the cells at the following times: 50 s, 1.25 μM; 200 s, 5 μM; 400 s, 10 μM; 600 s, 20 μM; and 800 s, 30 μM. For abbreviations see Figures 1, 3, and 4.

hibitory against L1210 cells, ET-18-OCH_3 elicited increased [Ca^{2+}] transients whereas ELL-12 had no effect. At all concentrations studied, ET-18-OCH_3 or ELL-12 did not decrease cell viability in serum containing media during the course of the Ca^{2+} experiments.

DNA fragmentation analysis. Figure 6 shows the effects of a 24-h incubation of ET-18-OCH_3 or ELL-12 on DNA fragmentation in L1210 and L1210/vmdr cells. In L1210 cells, DNA was fragmented in a ladder-like pattern, which may indicate programmed cell death (31). DNA ladder formation was detectable after the addition of 1 μM of both ET-18-OCH_3 (Fig. 6A) and ELL-12 (Fig. 6B) and became more evident with the additions of increasing drug concentrations in a dose-dependent manner. In contrast to the effects on L1210 cells, no DNA fragmentation was observed in L1210/vmdr cells after treatment with ET-18-OCH_3 (Fig. 6C) or ELL-12 up to concentrations of 20 μM (data not shown).

DISCUSSION

Although ET-18-OCH_3 has *in vivo* antitumor activity, a major dose-limiting toxicity is hemolysis (30). A number of reports document the advantages of associating chemotherapeutic agents in lipid complexes or liposomes to improve therapeutic efficacy, and such approaches have been productive and well-supported by clinical trials (17–19,32,33). ET-18-OCH_3 was associated with liposomes by Layton *et al.* (34) who showed a nonoptimized liposome formulation retained cytotoxicity and was selectively toxic to a leukemia cell line compared to a "normal" fibroblast cell line, but no investigation of the hemolytic effects of the formulation was described.

In this report we have compared the growth inhibitory and hemolytic effects of free and liposome-associated ET-18-OCH_3. The results indicate that association of ET-18-OCH_3 with liposomes decreased growth inhibition only 1.5–3.0-fold and the inhibitory effects were not strongly dependent on liposome composition. In contrast, hemolysis was highly dependent on liposome composition. When dioleoylphosphatidylcholine or dioleoylphosphatidylethanolamine was the major neutral phospholipid and 30 mol% cholesterol was included as a liposome component, hemolysis was decreased

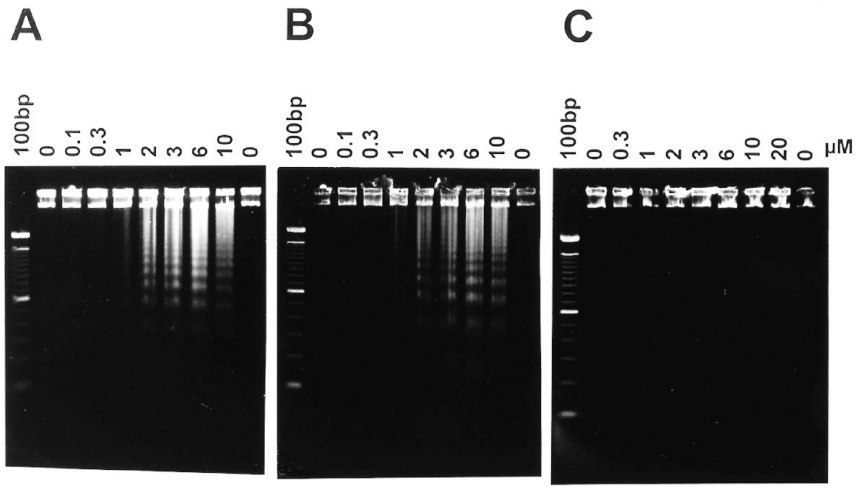

FIG. 6. Photographs of ethidium bromide-stained agarose gels after electrophoresis of DNA isolated from L1210 cells treated with different concentrations (μM) of ET-18-OCH_3 (A) or ELL-12 (B) for 24 h and L1210/vmdr cells treated with increasing concentrations of ET-18-OCH_3 (C) for 24 h. Far left lane in each panel is a 100 base pair (bp) ladder standard.

40 or more times compared to free ET-18-OCH_3. Decreased hemolysis was most likely related to the supramolecular organization of the lipid components where complementary packing in the lipid bilayer (35) provides a stable environment for ET-18-OCH_3, reducing the availability of ET-18-OCH_3 responsible for hemolysis and membrane lytic effects (21). Based on *in vitro* cytotoxicity and hemolysis data, we conclude that free ET-18-OCH_3 cannot be dosed at growth inhibitory concentrations without causing hemolysis, but optimized liposomal formulations can achieve growth inhibitory activity at concentrations 8–20-fold lower than those which cause hemolysis.

The various cell lines used had a 4–5-fold range in GI_{50} values to ET-18-OCH_3, and the relative contributions of cytotoxicity or cytostasis to growth inhibitory effects vary between cell types, as was found for L1210 and MCF7 cells. Different responses to growth inhibitory agents between cell types do not necessarily indicate that the mechanism of action of the agent differs between cell types. It may indicate, for example, that the relative balance of activating or deactivating enzymes varies between cell types. A 4–5-fold difference in drug sensitivity between six widely different cancer lines (human and mouse leukemias and human carcinomas including ovarian/breast/prostate/lung) is not great. However, for all cell types, the GI_{50} values for free ET-18-OCH_3 were paralleled by the GI_{50} values for the liposome formulations. This suggests that in each cell type, ET-18-OCH_3 from the liposome formulation was acting similarly to "free" ET-18-OCH_3. Association of ET-18-OCH_3 with liposomes therefore "protected" cells against acute membrane lytic effects while maintaining growth inhibitory properties. If membrane lysis results from insertion of ET-18-OCH_3 into the plasma membrane, then association of ET-18-OCH_3 with liposomes significantly attenuates these effects.

The different effects of ET-18-OCH_3 and ELL-12 on intracellular [Ca^{2+}] in L1210 cells also suggest that ET-18-OCH_3 and ELL-12 have different membrane-perturbing effects. Free ET-18-OCH_3 elicited both a transient intracellular [Ca^{2+}] increase at 1.25 μM and a permanent increase upon the addition of 5 μM in PBS, whereas ELL-12 did not modulate intracellular [Ca^{2+}] under the same conditions. The transient [Ca^{2+}] increase seen after the addition of 1.25 μM free ET-18-OCH_3 was not associated with a loss of cell viability and was probably due to Ca^{2+} liberation from intracellular sources and influx from the extracellular environment. Others have reported similar results with ET-18-OCH_3 in different cell lines in buffer systems (9,11,12). The permanent increase seen at 5 μM appeared to be associated with a loss of membrane integrity and therefore was not related to possible Ca^{2+}-modulated signal transduction. When changes in intracellular [Ca^{2+}] were determined in media containing 10% fetal bovine serum, free ET-18-OCH_3 also elicited transient [Ca^{2+}] increases, even though at higher concentrations than those required in PBS, but ELL-12 did not cause any changes in [Ca^{2+}] at any concentrations tested. The reduction in the membrane-perturbing effects of free ET-18-OCH_3 in the presence of serum was probably related to the efficient binding of ET-18-OCH_3 to albumin and high-density lipoproteins [Kelley *et al.* (4)]. In contrast to the different membrane effects of ET-18-OCH_3 and ELL-12 on L1210 cells, both affected DNA fragmentation of these cells in a similar concentration- and time-dependent manner, which implies apoptosis as a potential mechanism of action.

The results obtained in the cell lines selected for drug resistance were generally similar to those found for the nonresistant cells and, interestingly, in the sensitive/resistant cell line pairs; L1210, L1210/vmdr; P388, P388/adr; and MCF 7, MCF7/adr; both ET-18-OCH_3 and ELL-12 were equally potent, suggesting that ET-18-OCH_3 is not a substrate for the P-glycoprotein efflux pump. However, an unexpected result was found for the L1210/vmdr cells. Although the growth of these cells was affected by ET-18-OCH_3 and ELL-12 similarly, high drug concentrations did not induce ladder-like DNA fragmentation in L1210/vmdr cells. The differing drug responses may be indicative of multiple growth inhibitory mechanisms.

In conclusion, the results show that both free ET-18-OCH_3 and ELL-12 had similar growth inhibitory effects on cultured cells but, as indicated by the hemolysis and [Ca^{2+}] results, ELL-12 did not have the same direct membrane-perturbing effects as free ET-18-OCH_3. This suggests that in the growth inhibitory concentration range, the specific DNA fragmentation effects of ELL-12 were not a result of direct membrane damage. Thus, ELL-12 differs from ET-18-OCH_3 in that the specific *in vitro* growth inhibitory effects are retained, whereas nonspecific membrane-perturbing effects are reduced.

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In Vitro Effect of Garlic Powder Extract on Lipid Content in Normal and Atherosclerotic Human Aortic Cells

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ABSTRACT: In the present study, the mechanism of the *in vitro* effect of garlic powder extract (GPE) on lipid content of cultured human aortic cells was investigated. The addition of GPE abolished atherogenic blood serum-induced accumulation of free cholesterol, triglycerides, and cholesteryl esters in smooth muscle cells derived from uninvolved (normal) intima. In cells isolated from atherosclerotic plaque, GPE lowered these lipids. GPE inhibited lipid synthesis both in normal and atherosclerotic cells. It inhibited acyl-CoA:cholesterol acyltransferase activity that participates in the cholesteryl ester formation and stimulated cholesteryl ester hydrolase that degrades cholesteryl esters. This may explain the lipid reduction caused by GPE in atherosclerotic cells. GPE inhibited the uptake of modified low density lipoprotein and degradation of lipoprotein-derived cholesteryl esters, thus considerably reducing the intracellular accumulation of cholesteryl esters. This suggests the mechanism responsible for the prevention of lipid accumulation in aortic cells caused by atherogenic blood serum.

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Positive influence of garlic on cardiovascular risk factors has been reported (1,2). Recently we have found that aqueous extract of garlic powder (equivalent to Kwai®, Sapec®; Lichtwer Pharma, Berlin, Germany) possesses direct antiatherogenic-related (preventive) and antiatherosclerotic-related (therapeutic) effects at the arterial cell level *in vitro* (3). In primary culture, garlic powder extract (GPE) taken at the concentration range of 0.01–1 mg/mL inhibited proliferation of smooth muscle cells derived from uninvolved and atherosclerotic human aortic intima (3). In addition, GPE prevented intracellular accumulation of cholesterol caused by atherogenic blood serum of coronary heart disease patients as well as reduced cholesterol content in cells cultured from atherosclerotic plaques (3).

In the present study, we extended investigation of the *in vitro* effect of GPE on lipids of cultured human aortic cells. We have earlier shown that lipid accumulation in these cells is accompanied by stimulation of other cellular manifestation of atherosclerosis, namely, proliferation and extracellular matrix synthesis (4,5). Thus, investigation of GPE action on cellular lipid parameters is closely related to the study of the possible antiatherosclerosis effect of garlic.

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; CEH, cholesteryl ester hydrolase; FCS, fetal calf serum; GPE, garlic powder extract; HMG-CoA, hepatic hydroxymethylglutaryl CoA; LDL, low density lipoprotein(s).

MATERIALS AND METHODS

GPE. Aqueous GPE containing 3.58 mmol/L allicin and 0.184 mmol/L ajoene (6) was prepared according to Gebhardt (7). All concentration data are related to the initial amount of garlic powder per extraction volume. The garlic powder used is prepared immediately after harvesting of garlic cloves by careful air-drying. This powder is characterized by a very high content of sulfur-containing components and standardized to an allicin content of 1.3% and a capacity for liberation of allicin of 0.6%.

Cells. Human aortic subendothelial intimal smooth muscle cells were isolated from autopsy material obtained within 2–3 h after sudden death from 40- to 60-year-old men who had died of myocardial infarction. Usually human aorta contained uninvolved (grossly normal) areas and atherosclerotic lesions. Cells were harvested by enzymatic digestion of grossly normal part of aortic intima or atherosclerotic plaque with 0.1% collagenase for 3–4 h and seeded into 24-well culture plates. Cells were cultured for 7 d in Medium 199 containing 10% fetal calf serum (FCS), glutamine, and antibiotics. To identify smooth muscle cells and macrophages in primary cell cultures, cells were stained by immunoperoxidase reaction using monoclonal antibodies HHF-35 interacting with smooth muscle cells and HAM-56 recognizing macrophages (8). Primary cell cultures derived from grossly normal areas contained 87.1 ± 1.3% HHF-35-positive cells and 8.0 ± 1.5% HAM-56-positive cells; cell cultures from atherosclerotic plaques contained 81.0 ± 3.9% HHF-35-positive and 14.7 ± 2.8% HAM-56-positive cells (9). On the seventh day of cultivation, test agents were added, and then intracellular lipids were extracted after 24-h incubation. To induce lipid accumulation in cultured cells, 40% atherogenic serum was added to culture. Pooled atherogenic serum capable of inducing at least a 2-fold increase in total cholesterol level of cultured cells within 24-h incubation was prepared from blood taken from 10 normolipidemic, nondiabetic men with angiographically assessed coronary atherosclerosis. The characteristics of

atherogenic serum were described elsewhere (4,5). Protocols for cell isolation and cultivation as well as technical details of the experiment are presented elsewhere (10).

Lipoproteins. Low density lipoprotein (LDL, P = 1.019–1.063 g/mL) was isolated by sequential ultracentrifugation from blood plasma (0.1% EDTA) of coronary atherosclerosis patient according to Lindgren (11). Native and naturally occurring modified desialylated LDL were separated on lectin-agarose column as described elsewhere (12). After recentrifugation, LDL was labeled with [1,2 (*n*)-^{3}H]cholesteryl linoleate (Amersham International plc, Amersham, United Kingdom): 100 μCi of [^{3}H]cholesteryl linoleate was dissolved in 100 μL dimethylsulfoxide and mixed with 4–7 mg LDL in 2 mL 50 mM Tris-HCl (pH = 7.4) containing 1 M NaCl. LDL preparation was incubated 2 h at 40°C and dialyzed against 2000 vol of phosphate-buffered saline.

LDL metabolism. Normal and atherosclerotic cells were incubated for 24 h with 100 μg/mL of LDL labeled with [^{3}H]cholesteryl linoleate. After incubation, lipids were extracted from cells and culture media according to Hara and Radin (13) and Bligh and Dyer (14), respectively. Neutral lipids were separated by thin-layer chromatography using *n*-hexane/diethyl ether/ acetic acid (75:23:2, by vol) as solvent. Fractions of free and esterified cholesterol were scraped off and radioactivity was measured.

Lipids. Lipids from cells were extracted according to Hara and Radin (13). Neutral lipids were separated by thin-layer chromatography using two solvent systems (i) benzene/diethyl ether/ethanol/acetic acid (50:40:2:0.2, by vol) up to 6 cm from start line and then (ii) *n*-hexane/diethyl ether/acetic acid (90:10:1, by vol) in the same direction. Cholesteryl oleate, triolein, and cholesterol were used as standards. Phospholipids were separated by thin-layer chromatography using methyl acetate/*n*-propanol/chloroform/methanol/0.25% KCl (25:25:25:10:9, by vol) as solvent (15). Lipid content was quantitated by scanning densitometry using a dual-wavelength thin-layer chromato-scanner as described elsewhere (16).

Lipid metabolism. To investigate oleate incorporation into cellular lipids, human intimal cells were incubated during 24 h with 10 μCi/mL [^{14}C]oleate/albumin prepared according to Goldstein *et al.* (17). After incubation, lipids were extracted and separated as described above. Regions corresponding to phospholipids, triglycerides, and cholesteryl esters were scraped off, and radioactivity dissolved in toluene-based scintillation liquid was measured.

Enzyme activities. Freshly isolated normal and atherosclerotic cells were used for assay of enzyme activities. Cells were broken up by sonication (5 s) in 10 mM Tris-HCl, pH 7.0. Sonicates were centrifuged for 20 min at 4000 rpm to remove nuclei and debris.

To measure acyl-CoA:cholesterol acyltransferase (ACAT) activity, homogenate preparations were incubated 20 min with a reaction mixture containing 0.1 μCi [1-^{14}C]oleoyl-CoA (Amersham) in the presence or absence of garlic extract (1 mg/mL). Incubation was stopped by addition of chloroform/methanol (1:2, vol/vol). Cholesteryl esters were separated from other lipids by thin-layer chromatography on silica gel G using *n*-hexane/diethyl ether/acetic acid (75:23:2, by vol) for developing. Regions corresponding to cholesteryl esters were scraped into scintillation vials, and radioactivity was determined.

Cholesteryl ester hydrolase (CEH) activity was measured according to Haley *et al.* (18) using cholesteryl [1-^{14}C]oleate (Amersham) as a substrate.

Statistics. The significance of differences was evaluated using dispersion analysis methods and a BMDP statistical program package (19).

RESULTS

Lipids composition. Smooth muscle cells cultured from grossly normal intima and atherosclerotic plaque differed markedly in their contents of major lipid classes. Phospholipids, free cholesterol, cholesteryl esters, and triglycerides were much higher in atherosclerotic cells as compared to cells derived from normal intima (Table 1). Addition of serum from patients with coronary heart disease to a culture of normal cells induced an intracellular accumulation of all lipid classes except phospholipids. After 24 h of incubation with atherogenic serum, the content of cholesteryl esters increased 3.2-fold and those of triglycerides and free cholesterol 1.7-fold, which amounted to 60–80% of the corresponding lipid contents of cells cultured from atherosclerotic plaques (Table 1).

The addition of GPE abolished serum-induced accumulation of free cholesterol and triglycerides and reduced the accumulation of cholesteryl esters 2.8-fold (Table 1). Besides, in cells isolated from plaque, GPE lowered triglyceride, free cholesterol, and cholesteryl ester levels by 21, 31, and 35%, respectively (Table 1). GPE produced no significant effect on the total phospholipid content of smooth muscle cells cultured from grossly normal intima and atherosclerotic plaque (Table 1). In addition, it did not change the contents of sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and phosphatidylethanolamine both in normal and atherosclerotic cells (data not shown).

Lipids and lipoprotein metabolism. Incorporation of [^{14}C]oleate in the lipids of cells cultured from plaque was much greater than that in lipids of normal cells cultured in the presence of atherogenic serum, which may indicate an enhanced synthesis of phospholipids, triglycerides, and cholesteryl esters in atherosclerotic cells (Table 2). GPE decreased [^{14}C]oleate incorporation in the lipids of both normal and atherosclerotic cells.

LDL is the main source of free and esterified cholesterol that accumulates in arterial cells. Arterial smooth muscle cells can bind, internalize, and metabolize LDL. Intracellular metabolism of LDL-derived cholesteryl esters was studied using lipoprotein labeled with [^{3}H]cholesteryl linoleate. The major proportion of LDL-derived cholesteryl esters was degraded to free cholesterol and reappeared in the culture medium (Table 3). A portion of LDL circulating in patients with coro-

TABLE 1
Effect of GPE on Lipid Composition in Human Aortic Intimal Smooth Muscle Cells[a]

	Lipid content (μg/mg cell protein)			
	Phospholipids	Free cholesterol	Triglycerides	Cholesteryl esters
Normal cells				
Control	85.8 ± 3.9	10.5 ± 0.5	7.8 ± 0.7	10.4 ± 0.3
Atherogenic serum	92.3 ± 6.4	17.5 ± 0.7[b]	12.9 ± 0.7[b]	33.1 ± 1.2[b]
Atherogenic serum + GPE	84.4 ± 3.0	12.8 ± 0.4[c]	8.3 ± 0.3[c]	18.4 ± 0.7[b,c]
Atherosclerotic cells				
Control	136.1 ± 8.3	24.6 ± 1.2	16.0 ± 0.6	56.0 ± 3.3
+ GPE	131.8 ± 8.0	17.0 ± 1.0[b]	12.7 ± 0.7[b]	34.2 ± 1.5[b]

[a]Data represent mean of three determinations ± SEM from three separate experiments. Normal smooth muscle cells derived from grossly uninvolved intima and atherosclerotic cells derived from atherosclerotic plaque cultured in standard conditions with 10% fetal calf serum served as the control. Forty percentage of atherogenic blood serum pooled from patients with coronary atherosclerosis was added to cultured normal cells to induce intracellular lipid accumulation. Garlic powder extract (GPE) was used at concentration 1 mg/mL. Cells with all additives were incubated during 24 h.
[b]Significant difference from the control, $P < 0.05$.
[c]Significant difference from atherogenic serum, $P < 0.05$.

TABLE 2
Effect of GPE on [^{14}C]Oleate Incorporation into Intracellular Lipids[a]

	[^{14}C]Oleate incorporation (dpm/μg cell protein)		
	Phospholipids	Triglycerides	Cholesteryl esters
Normal cells			
Control, atherogenic serum	1922 ± 52	649 ± 22	127 ± 5
Atherogenic serum + GPE	1794 ± 66[b]	570 ± 18[b]	75 ± 5[b]
Atherosclerotic cells			
Control	6887 ± 324	1226 ± 51	274 ± 7
+ GPE	6145 ± 112[b]	795 ± 38[b]	201 ± 12[b]

[a]The cells were incubated with 1 μCi/mL [^{14}C]oleate during 24 h. For other details and abbreviation, see Table 1.

nary atherosclerosis is believed to be modified. In contrast to native LDL of healthy subjects, it induces intracellular accumulation of cholesteryl esters. Uptake and degradation of cholesteryl esters supplied to the cell by modified LDL was considerably greater than that of cholesteryl esters supplied by native LDL (Table 3). GPE reduced the internalization of cholesteryl esters supplied by native and modified LDL by 32 and 56%, respectively (Table 3). As a result, intracellular accumulation of cholesteryl esters caused by native and modified LDL was lowered almost 2- and 4-fold, respectively (Table 3).

Thus, GPE inhibits lipid synthesis both in normal and atherosclerotic cells. In addition, this agent suppresses LDL uptake and intracellular degradation of LDL-derived cholesteryl esters, leading to a decrease in intracellular accumulation of free and esterified cholesterol in arterial smooth muscle cells.

ACAT and CEH activities. The effect of GPE on the enzymes responsible for intracellular metabolism of cholesteryl

TABLE 3
Effect of GPE on the Metabolism of Low Density Lipoprotein-Derived Cholesteryl Esters in Intimal Smooth Muscle Cells[a]

	Intracellular (dpm/μg cell protein)		Extracellular (dpm/μg cell protein)
	Cholesteryl esters	Free cholesterol	Free cholesterol
Native LDL	92 ± 4	62 ± 3	4872 ± 119
Native LDL + GPE	49 ± 3[b]	47 ± 2[b]	3344 ± 203[b]
Modified LDL	194 ± 14	83 ± 5	7400 ± 503
Modified LDL + GPE	55 ± 6[b]	60 ± 4[b]	3295 ± 289[b]

[a]Smooth muscle cells cultured from uninvolved intima were incubated for 24 h with native and modified (desialylated) low density lipoproteins (LDL) (100 μg/mL) labeled with [^{3}H]cholesteryl linoleate.
[b]Significant effect of GPE (1 mg/mL), $P < 0.05$. For other details and other abbreviation, see Table 1.

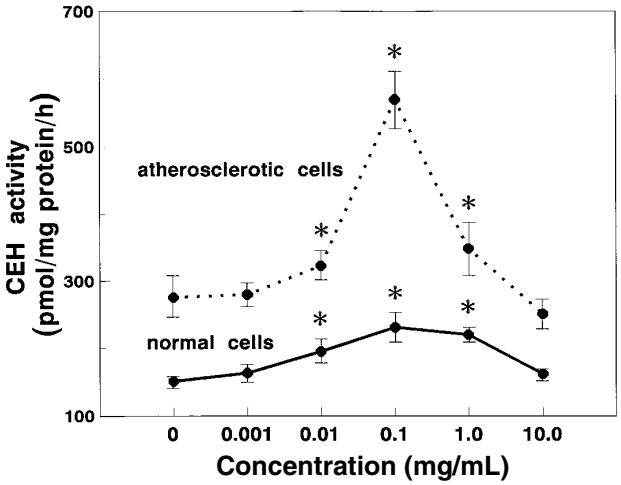

FIG. 1. Effect of garlic powder extract on acyl-CoA:cholesterol acyltransferase (ACAT) activity in aortic smooth muscle cells. Each data point represents the mean of three determinations ± SEM. Significant difference ($P < 0.05$) from the control (0 mg/mL garlic powder extract) is marked by an asterisk.

esters was studied by measuring the activities of ACAT, the enzyme involved in cholesteryl ester formation, and CEH, which hydrolyzes cholesteryl esters, in homogenates of normal and atherosclerotic cells.

The ACAT activity of atherosclerotic cells was 3-fold higher than that of normal cells (Fig. 1). At a concentration of 0.1 mg/mL and greater, GEP significantly decreased the enzyme activity in homogenates prepared from both atherosclerotic and normal cells (Fig. 1).

The CEH activity of atherosclerotic cells was almost 2-fold higher than that of normal cells (Fig. 2). At the concentration range of 0.01–1.0 mg/mL, GPE significantly increased the enzyme activity of both normal and atherosclerotic cells (Fig. 2). The dose dependence of the GPE effect had a bell-shaped pattern. The maximum effect was attained at 0.1 mg/mL GPE; at higher and lower concentrations the effect was less pronounced (Fig. 2).

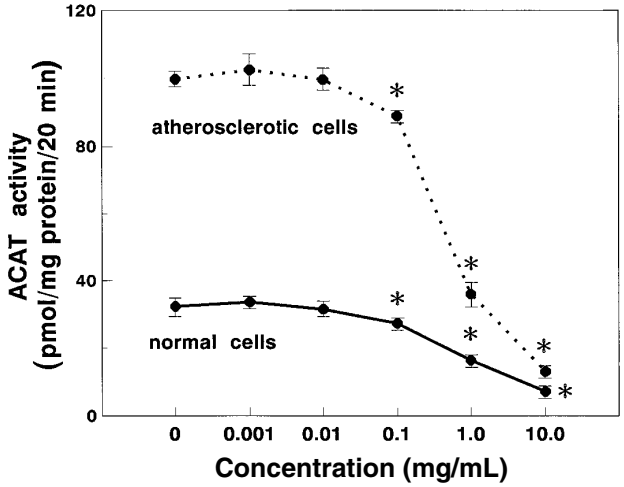

FIG. 2. Effect of garlic powder extract on cholesteryl ester hydrolase (CEH) activity in aortic smooth muscle cells. Details are the same as in the legends to Figure 1.

Thus, GPE inhibits ACAT that participates in the cholesteryl ester formation and stimulates CEH that degrades cholesteryl esters.

DISCUSSION

Garlic has been advocated for various diseases, including heart disease, headache, tumors, infections, and vascular disease. Evidence that garlic reduces cardiosvascular risk factors is accumulating (20). Spontaneous thrombocyte aggregation *in vitro* disappeared, the microcirculation increased, and plasma viscosity, diastolic blood pressure, and blood glucose concentration reportedly decreased as a result of garlic treatment (21). Garlic is also believed to exert beneficial effects on coagulation and serum lipid concentrations. Concentrations of triglycerides and total and LDL cholesterol fell while subjects ingested garlic, and the concentration of high density lipoprotein cholesterol increased (2).

The possibility of a direct influence of garlic on atherosclerosis has been discussed (22–24). Such putative antiatherosclerotic effects of garlic have been attributed to its hypolipidemic activity. Experimental and clinical data have demonstrated that garlic reduces blood cholesterol and LDL levels (2,24). The cholesterol-lowering effect of garlic possibly results from inhibition of hepatic HMG-CoA reductase (7,25).

In contrast to the above-cited studies, we examined not the hypolipidemic but the direct antiatherosclerotic-related and antiatherogenic-related effects of garlic, i.e., the ability of garlic to act directly on the atherosclerotic process in the vessel wall. Primary culture of human aortic cells, enzyme activities of these cells, and aqueous extract of garlic powder were used as a model. To investigate antiatherosclerotic-related effects, we used smooth muscle cells cultured from atherosclerotic plaques of human aorta. To study antiatherogenic-related effects, we imitated atherogenesis in primary cultures of smooth muscle cells derived from grossly uninvolved human aortic intima by adding atherogenic blood serum of patients with angiographically assessed coronary atherosclerosis. GPE decreased triglyceride, cholesteryl ester, and free cholesterol contents of cells cultured from atherosclerotic plaque and prevented atherogenic serum-induced accumulation of these lipids in cells cultured from grossly normal aorta, i.e., elicited direct antiatherosclerotic-related and antiatherogenic-related effects.

Inhibition of triglyceride and cholesteryl ester synthesis in aortic cells can be proposed as a tentative mechanism underlying the ability of GPE to reduce the lipid content of plaque-derived cells overloaded with intracellular fat, a potentially antiatherosclerotic effect. GPE inhibits ACAT and stimulates CEH, thus displaying a direct influence on synthesis and degradation of cholesteryl esters in the cell.

The concentration dependence of GPE stimulatory effect on CEH activity had a bell-shaped pattern, suggesting that

GPE contains agents that both stimulate and inhibit the enzyme activity. On the other hand, similar dose dependence was reported by Siegel *et al.* (6), who have shown that aqueous garlic extract and pure ajoene (a natural garlic product) induced a concentration-dependent hyperpolarization and relaxation in arterial vasculature. At high concentrations of ajoene and garlic extract, the concentration dependence of membrane potential becomes bell-shaped.

We did not examine the effects produced by individual components of GPE. Assuming that these components may exert an antiatherosclerotic effect, we plan to analyze them for such activity in the near future. According to Siegel *et al.* (6), the major components of GPE are allicin (3.58 mmol/L) and ajoene (0.184 mmol/L). Sendl *et al.* (25) have recently demonstrated that garlic extract and individual compounds ajoene, methylajoene, allicin, 2-vinyl-4H-1,3-dithiin, and diallydisulfide inhibited cholesterol synthesis on liver homogenate model.

A potentialy antiatherogenic effect of GPE was studied using cultured cells derived from grossly normal aorta and atherogenic blood serum of patients with coronary atherosclerosis. Previously, we have shown that atherogenic serum induces lipid accumulation in aortic cells which is accompanied by the major atherosclerosis-related manifestations at the cell level: stimulation of cell proliferation and increased extracellular matrix production (4,5). It was found that the modified LDL circulating in the blood of atherosclerotic patients is responsible for serum atherogenicity (4,26). Modification of patients' LDL includes a lower sialic acid content, higher density, smaller particle size, higher negative charge, etc. (27). This modified LDL was employed to induce lipid accumulation in cultured aortic cells. GPE inhibited the uptake of modified LDL and degradation of LDL-derived cholesteryl esters, thus reducing considerably the intracellular accumulation of cholesteryl esters. We believe that this mechanism is responsible for the potentially antiatherogenic effect of GPE on our model system.

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Breast Milk Fatty Acid Composition: A Comparative Study Between Hong Kong and Chongqing Chinese

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ABSTRACT: The fatty acids of milk samples obtained from 51 Hong Kong Chinese and 33 Chongqing Chinese (Si Chuan Province, China) were analyzed by gas–liquid chromatography. Compared with those of published data for Canadian and other Western countries, the Chinese milk from both Hong Kong and Chongqing contained higher levels of longer-chain polyunsaturated fatty acids, particularly docosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6). In contrast, the content of *trans* fatty acids in the Chinese milk was lower compared with those for Canadian and other Western countries. Longitudinally, the concentrations of 22:6n-3 and 20:4n-6 gradually decreased when lactation progressed from colostrum (week 1) to mature (week 6). Over the same interval, linoleic acid (18:2n-6) remained unchanged in Chongqing Chinese but significantly increased in Hong Kong Chinese. Unlike 18:2n-6, linolenic acid (18:3n-3) increased in Chongqing Chinese but remained unchanged in Hong Kong Chinese throughout the study. The total milk fat also increased with the duration of lactation. In addition, the milk of Chongqing Chinese had higher total milk fat than that of Hong Kong Chinese and Canadians. The content of erucic acid (22:1n-9) increased with the progression of lactation in Chongqing Chinese, indicating that there was a switch in dietary consumption from fats of animal origin to rapeseed oil when lactation reached week 6. The present study showed that Hong Kong and Chongqing Chinese had a different fatty acid profile in many ways, which largely reflected a different dietary habit and life-style in these two places.
Lipids 32, 1061–1067 (1997).

Human milk is considered the optimal form of nutrition for infants. The importance of n-6 and n-3 longer-chain polyunsaturated fatty acids (LCP) in human milk for normal brain development, especially during early life, has been emphasized in many studies (1–3). Both human milk and infant formula contain precursor essential fatty acids, linoleic acid (18:2n-6), and linolenic acid (18:3n-3). LCP such as arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3, DHA) are, however, naturally present in milk but they are generally absent in current infant formulas. These LCP are important for the structural development of the nervous system and are accumulated in large amounts in the developing brain and retina (4,5). There has been increasing interest in adding these LCP to infant formulas (6–10) because formula-fed preterm infants have lower erythrocyte levels of DHA than breast milk-fed preterm infants (1,2), and because the lower tissue levels of this fatty acid are associated with reduced retinal function (11) and reduced visual acuity of preterm infants (12).

The LCP composition in the tissues of growing infants is largely determined by the LCP content of the milk (1), which in turn partially reflects the fatty acid composition in the maternal diet (13–16). Although the fatty acid composition of milk has been reported in many countries (14–23), there is very limited information on fatty acid composition of milk in Chinese (24). The objective of the present study was therefore to investigate if fatty acid composition of milk in Hong Kong Chinese was significantly different from that in Chongqing Chinese, because the diets in the former are partially westernized and in the latter are still traditional.

MATERIALS AND METHODS

Sample and dietary data collection. Fifty-one and 33 lactating women from Hong Kong and Chongqing (Si Chuan Province, China), respectively, were recruited for this study. All mothers were on their usual diets; no dietary modifications were proposed. Donors were requested to express their milk manually after a feed on the assigned dates. The milk was collected at the first 3 d (colostrum milk) followed by 2, 4, and 6 wk after delivery. Milk (10–15 mL) was sampled each time in a bottle washed previously with deionized water. The milk samples were stored immediately at −20°C.

Dietary information was obtained by self-record on blank sheets over 3 d prior to each milk collection. At the beginning of the study, each subject was requested to record the amount of food and drink she consumed. In brief, the mothers were shown the common measuring kitchen utensils, for examples, standard bowl and tablespoon. They were also requested to record the brand name and size of commercially available products they consumed. The total energy and nutrient intake

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Abbreviations: LCP, longer-chain polyunsaturated fatty acids DHA, docosahexaenoic acid.

were computed using a food composition data base, Nutritionist IV (First Databank, San Bruno, CA). The protocol was approved by the Committee of Human Ethics, the Chinese University of Hong Kong, and signed consent was obtained from each milk donor.

Milk lipid extraction. Milk samples were thawed at room temperature (25°C). Fat from a 2-g sample was extracted using 15 vol of $CHCl_3$/MeOH (2:1, vol/vol) containing 1.5 mg/mL triheptadecanoin as an internal standard to quantify total milk fat (13). Another 2-g sample was extracted with the same solvent system but without addition of triheptadecanoin to determine the content of endogenous heptadecanoic acid in milk.

Fatty acid analysis. The milk lipids were converted to fatty acid methyl esters using a mixture of 14% BF_3/MeOH reagent (Sigma Chemical Co., St. Louis, MO) and toluene (1:1, vol/vol) at 90°C for 45 min under nitrogen. Fatty acid methyl esters were analyzed by gas–liquid chromatography using an SP-2560 flexible fused-silica capillary column (100 m × 0.25 mm i.d., 20 μm film thickness; Supelco, Inc., Bellefonte, PA) in a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame-ionization detector (Palo Alto, CA). Column temperature was programmed from 150 to 180°C at a rate of 0.5°C/min, and then to 210°C at a rate of 3°C/min. Injector and detector temperatures were 250 and 300°C, respectively. Hydrogen was used as the carrier gas at a head pressure of 20 psi. The gas chromatographic trace of the fatty acid methyl esters' profile of human milk is shown in Figure 1.

Statistics. Data were expressed as mean ± standard deviation. Analysis of variance followed by Student's *t*-test (two-tailed) where applicable was used for statistical evaluation of significant differences between groups.

TABLE 1
Dietary Intake of Lactating Women in Hong Kong and Chongqing, Sichuan, China[a]

	Hong Kong (n = 51)	Chongqing (n = 33)
Energy (kcal/d)	1809 ± 392	2686 ± 1030[b]
Carbohydrate (g/d)	244 ± 60	199 ± 125[b]
%kcal	54 ± 7	31 ± 15[b]
Protein (g/d)	77 ± 18	196 ± 99[b]
%kcal	17 ± 2	29 ± 7[b]
Fat (g/d)	58 ± 19	117 ± 58[b]
%kcal	28 ± 7	39 ± 9[b]
Food items (g/d)[c]		
Cooked rice	404	590
Eggs	50	340
Chicken	44	220
Pork	29	54
Fish	26	11
Vegetables	75	278
Fruits (apple and orange)	240	0
Milk	228	0
Bread	53	0

[a]Values are mean ± SD. The dietary data (for 3 d) were collected every time before the milk sample was collected.
[b]Means in the same row with different superscripts differ significantly at $P < 0.05$.
[c]No standard deviation could be given because the calculation was made by summing total consumption of each particular food item by all mothers and then dividing by the number of mothers.

RESULTS

The total energy intake between Chongqing and Hong Kong lactating women was significantly different (Table 1). The percentage energy intake from fat and protein was significantly higher in Chongqing than in Hong Kong women while the percentage energy derived from carbohydrate was higher in the latter. Traditionally, Chongqing Chinese stay and eat at home after delivery for at least a month to replenish their nutrient stores. Their dietary fat was usually of animal origin including chicken and pork together with 4–10 eggs/d (Table 1). They consumed no dairy products and fruits. In contrast, Hong Kong Chinese lactating women consumed milk and other dairy products, more fruits, and lesser amounts of vegetables than the Chongqing women (Table 1).

The average fatty acid composition of milk pooled over all stages of lactation in Chongqing and Hong Kong Chinese lactating women is shown in Table 2. For comparison, the data published by us for Canadian women are also shown in Table 2 (13). LCP including 22:6n-3 and 20:4n-6 in the milk of both Hong Kong and Chongqing Chinese were higher than those in the milk from Canadian women and milk from the majority of women from other Western countries. In contrast, the milk from both Hong Kong and Chongqing Chinese had lower contents of *trans* fatty acids than that from women of Canada and other Western countries. However, the total *trans* fatty acids in the milk of Hong Kong Chinese were significantly higher than in that of Chongqing Chinese.

Several differences in the milk fatty acid profiles were observed between Hong Kong Chinese and Chongqing Chinese women (Table 2). The milk in Hong Kong Chinese women contained more n-6 fatty acids, including mainly 18:2n-6. Compared with that of the Chongqing Chinese women, the milk of the Hong Kong Chinese women had less *cis*-monounsaturated fatty acids including 18:1n-9, 16:1n-7, 20:1n-9, and 22:1n-9. Among saturates, 16:0, 18:0, 12:0, and 10:0 were lower while 14:0 was higher in the milk of Hong Kong Chinese women than these in Chongqing Chinese women (Table 2). However, there appeared to be no difference in total n-3 fatty acids between these two places.

As lactation progressed in Chongqing and Hong Kong Chinese women, the milk n-6 LCP decreased although their precursor fatty acid, 18:2n-6, remained unchanged in Chongqing Chinese women or significantly increased in Hong Kong Chinese women (Table 3). These n-6 LCP include 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, and 22:5n-6 (Table 3). Similarly, the milk n-3 LCP including 22:6n-3 and 22:5n-3 also decreased gradually with the duration of lactation although the precursor fatty acid, 18:3n-3, increased in the milk of Chongqing Chinese women. Among saturated fatty acids, 16:0 and 14:0 progressively decreased in the milk of both Chongqing and Hong Kong Chinese women. In contrast, 18:0 and 12:0 increased in the milk of Hong Kong Chinese women, but they decreased in

TABLE 2
Average Fatty Acid Composition (wt% total fatty acids) of Milk Samples Pooled over All Stages of Lactation in Hong King and Chongqing Chinese Women Compared with That of Canadian Women[a]

Fatty acids	Chongqing (n = 33)	Hong Kong (n = 51)	Canadian[b] (n = 198)
Saturated			
10:0	0.81 (0.40)[b]	0.53 (0.38)[c]	1.39 (0.59)[a]
12:0	4.45 (2.14)[b]	4.23 (2.15)[b]	5.68 (2.01)[a]
14:0	4.22 (2.16)[c]	5.50 (2.01)[b]	6.10 (1.73)[a]
15:0	0.16 (0.05)[c]	0.31 (0.10)[b]	0.37 (0.12)[a]
16:0	22.79 (2.01)[a]	21.29 (2.27)[b]	18.30 (2.25)[b]
17:0	0.25 (0.07)	0.32 (0.08)	0.32 (0.08)
18:0	7.74 (1.39)[a]	5.86 (0.98)[c]	6.15 (0.97)[b]
20:0	0.20 (0.05)[a]	0.20 (0.15)[a]	0.15 (0.09)[b]
Cis-monounsaturated			
14:1n-5	.01 (0.01)[b]	0.01 (0.02)[b]	0.28 (0.08)[a]
16:1n-7	2.46 (0.63)[a]	2.23 (0.59)[b]	2.27 (0.56)[b]
16:1n-9	0.59 (0.08)[a]	0.56 (0.09)[a]	0.41 (0.13)[b]
17:1n-7	0.19 (0.03)	0.22 (0.07)	0.21 (0.06)
18:1n-9	37.15 (3.28)[a]	34.40 (3.90)[b]	30.65 (2.66)[c]
Total other 18:1*c*	2.25 (0.30)[b]	2.02 (0.78)[b]	4.22 (0.38)[a]
20:1n-9	0.97 (0.41)[a]	0.79 (0.29)[a]	0.39 (0.13)[c]
20:1n-7	0.01 (0.01)[b]	0.03 (0.04)[b]	0.14 (0.09)[a]
22:1n-9	0.58 (0.55)[a]	0.16 (0.14)[b]	0.02 (0.03)[c]
n-6 Polyunsaturated			
18:2n-6	10.44 (2.45)[b]	15.80 (3.01)[a]	10.47 (2.62)[b]
18:3n-6	0.11 (0.08)[a]	0.15 (0.09)[a]	0.08 (0.06)[b]
20:2n-6	0.44 (0.14)[b]	0.68 (0.32)[a]	0.17 (0.37)[c]
20:3n-6	0.37 (0.12)[a]	0.41 (0.16)[a]	0.26 (0.09)[b]
20:4n-6	0.76 (0.24)[a]	0.61 (0.18)[a]	0.35 (0.11)[b]
22:4n-6	0.17 (0.09)[c]	0.23 (0.20)[a]	0.04 (0.05)[c]
22:5n-6	0.10 (0.08)[a]	0.09 (0.11)[a]	0.01 (0.02)[b]
n-3 Polyunsaturated			
18:3n-3	1.17 (0.53)	1.24 (0.54)	1.16 (0.37)
20:5n-3	0.04 (0.05)[b]	0.08 (0.09)[a]	0.05 (0.05)[b]
22:5n-3	0.22 (0.08)[a]	0.23 (0.14)[a]	0.08 (0.05)[b]
22:6n-3	0.54 (0.20)[a]	0.56 (0.23)[a]	0.14 (0.10)[b]
Trans			
14:1*t*	<0.01	<0.01	0.09 (0.05)
16:1*t*	<0.01	<0.01	0.18 (0.08)
Total 18:1*t*	0.20 (0.04)[c]	0.70 (0.51)[b]	5.87 (2.52)[a]
Total 18:2*t*	0.02 (0.03)[c]	0.18 (0.14)[b]	0.94 (0.42)[a]
Total saturated	40.49 (5.14)[a]	38.07 (4.56)[b]	38.50 (2.94)[b]
Total C_{10}–C_{13}	5.26 (2.43)[b]	4.76 (2.45)[c]	7.11 (2.53)[a]
Total C_{14}–C_{16}	26.80 (3.84)[a]	26.79 (3.14)[a]	24.77 (3.26)[b]
Total n-6	12.38 (2.45)[b]	17.97 (2.94)[a]	11.39 (3.18)[b]
Total n-3	2.12 (0.55)[a]	2.17 (0.74)[a]	1.49 (0.44)[b]
Total n-6 LCP	1.83 (0.51)[a]	2.02 (0.74)[a]	0.83 (0.28)[b]
Total n-3 LCP	0.96 (0.27)[c]	0.92 (0.45)[a]	0.33 (0.24)[b]
Total *trans*	0.22 (0.06)[c]	0.88 (0.61)[b]	7.19 (3.03)[a]
Total milk fat (g/L)	38.1 (17.1)[a]	32.4 (21.9)[b]	31.6 (9.4)[b]

[a]Data are mean (SD). Means in the same row with different superscripts (a–c) differ significantly at $P < 0.01$. Abbreviation: LCP, longer-chain polyunsaturated fatty acids.
[b]Adapted from Reference 13; milk samples were obtained during 3–4 wk of lactation.

that of Chongqing Chinese women (Table 3). With *cis*-monounsaturated fatty acids, 18:1n-9 remained unchanged in the milk of Hong Kong Chinese women, but it increased in that of Chongqing Chinese women (Table 3). In the milk of Chongqing Chinese women, 20:1n-9 and 22:1n-9 increased but they decreased in that of Hong Kong Chinese women.

The total milk fat was determined by the amount of triheptadecanoin added. As can be seen in Table 2, the total milk fat was very similar for Hong Kong Chinese and Canadian women, but it was significantly higher in the milk of Chongqing Chinese women. The total milk fat increased significantly when lactation progressed from colostrum (week 1) to week 6 in both Chongqing and Hong Kong Chinese women.

DISCUSSION

Chongqing and Hong Kong Chinese have different dietary habits and life-styles. Populations in Chongqing have a more

TABLE 3
Milk Fatty Acid Composition (wt% total fatty acids) of Hong Kong and Chongqing Chinese Women at Different Stages of Lactation[a]

Fatty acids		Colostrum	Week 2	Week 4	Week 6
Saturated					
10:0	Chongqing	0.69 (0.39)[b]	0.94 (0.45)[a]	0.88 (0.36)[a,b]	0.76 (0.33)[b]
	Hong Kong	0.33 (0.29)[b,*b*]	0.66 (0.31)[a,*b*]	0.61 (0.39[a,b,*b*]	0.61 (0.45)[a,b]
12:0	Chongqing	4.85 (2.47)[a,b]	5.21 (2.55)[a]	4.08 (1.34)[a,b]	3.46 (0.97)[b]
	Hong Kong	3.01 (1.93)[b,*b*]	5.32 (1.76)[a]	4.73 (1.78)[a]	4.30 (2.28)[a]
14:0	Chongqing	5.22 (2.60)[a]	4.60 (2.33)[a,b]	3.53 (1.17)[b]	3.19 (1.05)[b]
	Hong Kong	5.35 (2.02)[a,b]	6.04 (1.94)[a,*b*]	5.48 (2.03)[a,b,*b*]	4.94 (1.83)[b,*b*]
15:0	Chongqing	0.18 (0.04)[a]	0.16 (0.04)[b]	0.16 (0.03)[b]	0.14 (0.06)[b]
	Hong Kong	0.32 (0.09)[*b*]	0.31 (0.11)[*b*]	0.30 (0.10)[*b*]	0.30 (0.10)[*b*]
16:0	Chongqing	23.25 (1.68)[a]	23.30 (1.61)[a]	23.08 (1.87)[a]	21.45 (2.23)[b]
	Hong Kong	22.64 (2.47)[a]	20.66 (2.02)[b,*b*]	20.68 (1.58)[b,*b*]	20.33 (1.57)[b,*b*]
17:0	Chongqing	0.28 (0.08)	0.25 (0.06)	0.26 (0.06)	0.24 (0.07)
	Hong Kong	0.33 (0.09)	0.32 (0.08)	0.31 (0.07)	0.31 (0.06)
18:0	Chongqing	7.02 (1.19)[b]	8.12 (1.46)[a]	7.95 (1.41)[a]	8.03 (1.14)[a]
	Hong Kong	5.98 (1.07)[*b*]	5.73 (0.97)[*b*]	5.77 (0.74)[*b*]	5.96 (1.02)[*b*]
20:0	Chongqing	0.19 (0.05)	0.20 (0.05)	0.19 (0.03)	0.22 (0.05)
	Hong Kong	0.18 (0.09)	0.19 (0.07)	0.20 (0.09)	0.21 (0.07)
Cis-monounsaturated					
16:1n-7	Chongqing	2.43 (0.53)	2.42 (0.62)	2.75 (0.67)	2.28 (0.61)
	Hong Kong	1.85 (0.38)[b,*b*]	2.44 (0.55)[a]	2.51 (0.52)[a]	2.34 (0.64)[a]
16:1n-9	Chongqing	0.63 (0.07)[a]	0.61 (0.06)[a,b]	0.59 (0.06)[b]	0.52 (0.08)[c]
	Hong Kong	0.59 (0.06)[a,*b*]	0.54 (0.09)[b,*b*]	0.55 (0.10)[b]	0.53 (0.08)[b]
17:1n-7	Chongqing	0.19 (0.03)	0.18 (0.02)	0.19 (0.02)	0.21 (0.04)
	Hong Kong	0.19 (0.07)	0.23 (0.05)	0.26 (0.07)	0.23 (0.06)
18:1n-9	Chongqing	36.27 (3.79)[b]	36.51 (3.42)[b]	37.79 (2.65)[a,b]	38.36 (2.52)[a]
	Hong Kong	34.60 (4.76)	34.02 (3.63)[a,*b*]	34.17 (3.39)[*b*]	34.92 (2.63)[*b*]
Other 18:1*c*	Chongqing	2.32 (0.28)[a]	2.15 (0.24)[b]	2.24 (0.32)[a,b]	2.27 (0.31)[a,b]
	Hong Kong	2.36 (0.92)	2.07 (0.75)	2.00 (0.73)	2.35 (0.28)
20:1n-9	Chongqing	0.89 (0.25)[b]	0.77 (0.23)[b]	0.86 (0.32)[b]	1.39 (0.44)[a]
	Hong Kong	0.99 (0.27)[a]	0.73 (0.25)[b]	0.66 (0.19)[b,*b*]	0.68 (0.21)[b,*b*]
22:1n-9	Chongqing	0.37 (0.26)[b]	0.28 (0.31)[b]	0.41 (0.24)[b]	1.29 (0.52)[a]
	Hong Kong	0.25 (0.18)[a,*b*]	0.13 (0.08)[b,*b*]	0.12 (0.10)[b,*b*]	0.10 (0.08)[b,*b*]
n-6 Polyunsaturated					
18:2n-6	Chongqing	10.30 (2.18)	9.76 (1.78)	10.43 (2.21)	11.34 (3.17)
	Hong Kong	14.85 (2.84)[c,*b*]	15.47 (2.79)[b,c,*b*]	16.75 (2.89)[b,*b*]	17.10 (2.95)[a,*b*]
18:3n-6	Chongqing	0.11 (0.10)	0.09 (0.06)	0.12 (0.06)	0.14 (0.03)
	Hong Kong	0.09 (0.07)[b]	0.18 (0.10)[a,*b*]	0.18 (0.09)[a,*b*]	0.17 (0.07)[a,*b*]
20:2n-6	Chongqing	0.51 (0.09)[a]	0.40 (0.13)[b]	0.39 (0.09)[b]	0.43 (0.09)[b]
	Hong Kong	0.95 (0.23)[a,*b*]	0.60 (0.39)[b,*b*]	0.51 (0.09)[b,*b*]	0.48 (0.0)[b,*b*]
20:3n-6	Chongqing	0.48 (0.09)[a]	0.39 (0.13)[b]	0.34 (0.07)[c]	0.26 (0.08)[d]
	Hong Kong	0.50 (0.17)[a]	0.39 (0.13)[b]	0.36 (0.14)[b]	0.34 (0.11)[b,*b*]
20:4n-6	Chongqing	0.84 (0.24)[a]	0.84 (0.20)[a,b]	0.79 (0.23)[b]	0.53 (0.16)[c]
	Hong Kong	0.71 (0.19)[a,*b*]	0.60 (0.17)[a,b,*b*]	0.56 (0.16)[b,c,*b*]	0.52 (0.10)[c]
22:4n-6	Chongqing	0.23 (0.09)[a]	0.19 (0.07)[a,b]	0.16 (0.06)[b]	0.10 (0.05)[c]
	Hong Kong	0.39 (0.25)[a,*b*]	0.16 (0.08)[b]	0.13 (0.09)[b]	0.14 (0.08)[b]
22:5n-6	Chongqing	0.12 (0.08)[a]	0.11 (0.08)[a]	0.10 (0.07)[a]	0.04 (0.03)[b]
	Hong Kong	0.11 (0.05)	0.07 (0.06)[*b*]	0.08 (0.07)	0.08 (0.07)
n-3 Polyunsaturated					
18:3n-3	Chongqing	0.85 (0.28)[c]	0.96 (0.24)[c]	1.21 (0.38)[b]	1.73 (0.61)[a]
	Hong Kong	1.27 (0.42)[*b*]	1.15 (0.57)	1.23 (0.64)	1.37 (0.50)[*b*]
20:5n-3	Chongqing	0.01 (0.03)	0.02 (0.03)	0.04 (0.04)	0.03 (0.05)
	Hong Kong	0.07 (0.14)[a]	0.05 (0.01)[a,b]	0.05 (0.13)[a,b]	0.02 (0.04)[b]
22:5n-3	Chongqing	0.23 (0.09)	0.22 (0.06)	0.23 (0.10)	0.20 (0.06)
	Hong Kong	0.28 (0.17)[a]	0.22 (0.13)[a,b]	0.19 (0.10)[b]	0.18 (0.07)[b]
22:6n-3	Chongqing	0.64 (0.16)[a]	0.61 (0.17)[a,b]	0.54 (0.18)[b]	0.35 (0.14)[c]
	Hong Kong	0.60 (0.24)[a]	0.58 (0.25)[a]	0.53 (0.23)[a,b]	0.48 (0.15)[b,*b*]
Trans					
18:1*t*	Chongqing	0.18 (0.04)[b]	0.20 (0.04)[b]	0.20 (0.02)[b]	0.23 (0.05)[a]
	Hong Kong	0.66 (0.28)[*b*]	0.71 (0.28)[*b*]	0.69 (0.54)[*b*]	0.75 (0.51)[*b*]
18:2*t*	Chongqing	0.01 (0.02)	0.02 (0.02)	0.02 (0.03)	0.02 (0.03)
	Hong Kong	0.15 (0.012)[*b*]	0.20 (0.16)[*b*]	0.20 (0.16)[*b*]	0.21 (0.12)[*b*]

(continued)

TABLE 3 (continued)

Fatty acids		Colostrum	Week 2	Week 4	Week 6
Total saturated	Chongqing	41.35 (5.62)[a]	42.63 (4.90)[a]	39.95 (3.91)[a,b]	37.37 (3.90)[b]
	Hong Kong	37.94 (4.55)[b]	39.08 (4.43)[b]	37.98 (4.81)	36.80 (3.99)
Total C_{10}–C_{13}	Chongqing	5.55 (2.78)[a]	6.15 (2.93)[a]	4.96 (1.63)[a,b]	4.22 (1.16)[b]
	Hong Kong	3.34 (2.17)[b,b]	5.97 (1.96)[a]	5.34 (2.06)[a]	4.91 92.65)[a]
Total C_{14}–C_{16}	Chongqing	28.28 (3.01)[a]	27.89 (2.53)[a,b]	26.61 (2.43)[b]	24.65 (2.84)[c]
	Hong Kong	28.00 (3.25)[a]	26.71 (3.04)[a,b]	26.17 (3.05)[b]	25.28 (1.99)[b]
Total n-6	Chongqing	12.58 (2.29)	11.78 (1.88)	12.33 (2.25)	12.83 (3.09)
	Hong Kong	17.59 (2.79)[b]	17.47 (2.99)[b]	18.58 (2.86)[b]	18.82 (2.84)[b]
Total n-3	Chongqing	1.87 (0.39)[c]	1.96 (0.35)[b,c]	2.19 (0.51)[b]	2.54 (0.65)[a]
	Hong Kong	2.33 (0.61)[b]	2.08 (0.85)	2.05 (0.83)	2.12 (0.60)[b]
Total n-6 LCP	Chongqing	2.18 (0.44)[a]	1.93 (0.43)[b]	1.78 (0.42)[b]	1.36 (0.33)[c]
	Hong Kong	2.65 (0.78)[a]	1.82 (0.55)[b]	1.65 (0.37)[b,c]	1.55 (0.29)[c]
Total n-3 LCP	Chongqing	1.02 (0.26)[a]	1.00 (0.23)[a]	0.98 (0.29)[a]	0.81 (0.23)[b]
	Hong Kong	1.07 (0.48)[a]	0.93 (0.47)[a,b]	0.82 (0.41)[b]	0.75 (0.25)[b]
Total *trans*	Chongqing	0.19 (0.25)[b]	0.21 (0.04)[b]	0.22 (0.04)[b]	0.26 (0.05)[a]
	Hong Kong	0.81 (0.30)[b]	0.91 (0.79)[b]	0.89 (0.67)[b]	0.97 (0.49)[b]
Total milk fat (g/L)	Chongqing	30.1 (11.8)[b]	39.7 (14.9)[a]	41.3 (19.7)[a]	43.5 (18.6)[a]
	Hong Kong	23.9 (16.1)[b]	38.7 (22.6)[a]	33.8 (19.8)[a]	37.3 (23.6)[a]

[a]Data are means (SD). Means in the same row with different superscripts (a–d) differ significantly at $P < 0.01$.
[b]Difference between Chongqing and Hong Kong is significant at $P < 0.01$.

traditional life-style where people have to rely on foods provided by the nearby farms. The general belief is that for the first month postpartum, women have to replenish their body nutrient stores by consuming more eggs, chicken, and pork. Eating more than eight eggs and 200 g chicken meat per day is not uncommon during the first month after delivery. This unique dietary habit leads to a very high intake of protein and fat in Chongqing women. Fruits are considered to be "cold" food and not commonly consumed during the first month. Milk and dairy products are never food items during pregnancy and lactation. In Hong Kong, however, people have a sedentary life-style and the diet is partly westernized. On average, a lactating Hong Kong Chinese woman drinks a glass of milk and eats a slice of bread for breakfast. During pregnancy and lactation, Hong Kong women consume more fruits than Chongqing Chinese women (Table 1). Furthermore, the amounts of rice, eggs, and meat eaten are not as much as for Chongqing Chinese women because of a lower requirement of energy expenditure in physical activity. This dietary habit clearly contrasts to what was observed for Chongqing women (Table 1). As shown in Table 1, the total energy intake in Hong Kong lactating women was only 1809 kcal/d which is consistent with their desire to get back to their prepregnant body shapes by consuming less total energy.

The present study showed that 22:6n-3 and 20:4n-6 levels in Chinese milk were higher than what has been reported in the majority of Western countries (20,21,25–27). The present values were similar to those in Japan (7), Africa (28), and other Asian countries (29). In some cases, the milk content of 22:6n-3 in Chinese women from fishery areas could reach 2.8% of the total milk fat (24). High seafood intake may contribute to the higher n-3 LCP content in human milk from Hong Kong relative to that from Canada and other Western countries. In contrast, the unique dietary characteristic of consuming more than eight eggs per day may contribute to a higher n-3 LCP content in milk from Chongqing lactating women. It is well known that eggs contain a significant amount of 22:6n-3 ranging from 0.9 to 6.6% of total lipids (30). In addition, the intake of chicken and pork would contribute to a higher 20:4n-6 content in milk from Chongqing Chinese women. The 20:4n-6 and 22:6n-3 contents of foods consumed by the women in this study have not been reported to our knowledge. Furthermore, a higher LCP content in the Chinese milk may be due in part to a very low *trans* fatty acid intake in these two places relative to Western countries. It has been suggested that dietary *trans* fatty acids may inhibit chain elongation and desaturation of 18:2n-6 and 18:3n-3 to form 20:4n-6 and 22:6n-3, respectively, in both maternal liver and mammary glands (31). As seen in Table 1, the two precursor fatty acids, 18:2n-6 and 18:3n-3, were similar in the milk of Chongqing Chinese and Canadian women but 22:6n-3 and 20:4n-6 in the former were much higher than in the latter. In contrast, the total *trans* fatty acid content in the Canadian samples was 32-fold higher than that in the Chongqing Chinese samples.

Our results illustrate a marked difference in dietary 20:4n-6 between Hong Kong and Chongqing Chinese lactating women although the milk content of this fatty acid was very similar (Table 2). Eggs and meats are generally a rich source of 20:4n-6. By assuming the fatty acid content of eggs

and meats consumed by the Chinese women is similar to that reported by Mann *et al.* (32), the Chongqing Chinese women would most likely have been consuming very high levels of 20:4n-6 (700–800 mg/d) compared with the Hong Kong Chinese women (120–140 mg/d). Together with observations made by Mantzioris *et al.* (33) and Makrides *et al.* (34), the present results suggest that breast milk 20:4n-6 may not be sensitive to maternal diet. In fact, it has been known that the breast milk 20:4n-6 level in vegan women (no or very little dietary 20:4n-6) is very similar to that of omnivorous women (35). However, it is important to conduct analyses of local foods to establish a reliable local food composition database before this finding can be confirmed.

The fatty acid composition in the milk of Hong Kong and Chongqing Chinese did not remain constant throughout the 6 wk of lactation. The 22:6n-3 and 20:4n-6 levels decreased gradually as lactation progressed from week 1 to week 6. This observation was in agreement of that of Gibson and Kneebone (18), who showed that 22:6n-3 and 20:4n-6 were significantly decreased from colostrum (22:6n-3, 0.64%; 20:4n-6, 0.71%) to mature milk (22:6n-3, 0.32%; 20:4n-6, 0.31%) in Australian lactating women. It was also evident that these fatty acids in mature milk decreased with the duration of lactation (25). However, the results of the present study were in contrast to those of Clark *et al.* (36), who showed that the total fatty acid composition in the milk of 10 lactating women remained constant throughout 16 wk of lactation. In their study, LCP appeared to decrease with time of lactation although a statistical significance was not found probably because the number of the subjects was relatively small compared to that in the present study. It is likely that the change in milk fatty acid composition is largely due to change in maternal diet as lactation progresses (14,36).

Differences in dietary patterns are also reflected by examining the content of individual fatty acids in the milk from Chongqing and Hong Kong. Firstly, 18:2n-6 in the milk from Hong Kong women was higher than that from Chongqing women. This may be due to the higher consumption of eggs and animal fats in the latter and a higher consumption of corn oil in the former during the first month of lactation. Secondly, eicosapentaenoic acid (20:5n-3) was higher in the milk of Hong Kong Chinese women than that of Chongqing Chinese women. This is possibly because Hong Kong lactating women consume more fish and fish soup presumably for inducing milk letdown. Thirdly, erucic acid (22:1n-9) and 18:3n-3 in the milk of Chongqing Chinese women increased as lactation progressed (Table 3). This is attributed to a change in diet from week 4 to week 6 in Chongqing lactating women. After one month of lactation, the Chongqing lactating women switched from a high animal fat diet to a normal diet, the same as the rest of the family. The 22:1n-9 and 18:3n-3 levels were therefore increased in the milk because high-erucic rapeseed oil is one of the major vegetable oils consumed in the Chongqing population.

In summary, the results from the present study are unique because there are few longitudinal and cross-cultural reports available of the fatty acids composition of the milk. The milk fatty acid composition, which reflects that in diets, is different in many ways between Chongqing and Hong Kong Chinese lactating women. Compared with that in the milk of women from Canada or other Western countries, the Chinese milk is characterized by having higher LCP and lower *trans* fatty acids contents. The relative higher *trans* fatty acids contents in the milk of Hong Kong Chinese than Chongqing Chinese may reflect the use of partially hydrogenated vegetable oils and dairy products in Hong Kong.

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Comparative Hypocholesterolemic Effects of Six Dietary Oils in Cholesterol-Fed Rats After Long-Term Feeding

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ABSTRACT: Rats (8 wk of age) fed a conventional diet were shifted to diets containing 10% *Oenothera biennis* Linn oil (OBLO, linoleic acid + γ-linolenic acid) from a wild plant, evening primrose oil (EPO, linoleic acid + γ-linolenic acid) from a cultivated plant, bio-γ-linolenic acid oil from mold (BIO, palmitic acid + oleic acid + linoleic acid + γ-linolenic acid), safflower oil (linoleic acid), palm oil (PLO, palmitic acid + oleic acid + linoleic acid), or soybean oil (linoleic acid + α-linolenic acid) with 0.5% cholesterol for 13 wk. Though there were no significant differences in the food intake among the groups, the body weight gain of the OBLO group was significantly lower than that of other groups except for the BIO and PLO groups, and that of the EPO and SBO groups were the highest among the groups. The liver weight of the OBLO group was significantly lower than that of other groups, and that of the PLO group was the highest among the groups. The serum total cholesterol and very low density lipoprotein (VLDL) + intermediate density lipoprotein (IDL) + low density lipoprotein (LDL) cholesterol concentrations of the OBLO and EPO groups were consistently lower than those in the other groups. However, those of the BIO group were higher than those in the OBLO and EPO groups. The liver cholesterol concentration of the PLO group was the highest among all groups except for the EPO group. The fecal neutral sterol and bile acid extraction of the BIO group tended to increase compared to the other groups. The results of this study demonstrate that OBLO and EPO inhibit the increasing of serum total cholesterol and VLDL + IDL + LDL-cholesterol concentrations in the presence of excess cholesterol in the diet compared with the other dietary oils.
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It is widely accepted that diets rich in linoleic acid (LA, 18:2n-6) reduce plasma cholesterol levels (1,2). There is no doubt that when LA replaces saturated fatty acids in the diet, the major portion of cholesterol lowering occurs in the low density lipoprotein (LDL) fraction (3). However, it also is reported that dietary high-linoleate safflower oil (SFO) is not hypocholesterolemic in aged mice compared with perilla oil and fish oil (n-3 fatty acids) after long-term feeding (4). Although it has been reported that n-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), suppress 3-hydroxy-3-methylglutaryl CoA reductase (5–7) and retard very low density lipoprotein (VLDL) secretion, resulting in decreased LDL (7), the mechanisms involved in the hypocholesterolemic effect of polyunsaturated fats have not been clearly defined.

Fukushima *et al.* (8) reported that *Oenothera biennis* Linn oil (OBLO) containing about 71% LA and 14% γ-linolenic acid (GLA) has a hypocholesterolemic function compared with soybean oil (SBO) containing about 51% LA and 9% α-linolenic acid (ALA), sunflower oil containing about 64% LA, high-oleic safflower oil (SFO) containing about 40% oleic acid and 50% LA, and mixed oil containing about 23% LA and 7% ALA after 6-wk feeding. The purpose of the present study is to investigate the effect of OBLO containing about 14% GLA from a wild plant on the lipid metabolism in rats in comparison with evening primrose oil (EPO) (Efamol) containing about 9% GLA from a cultivated plant, about 23% GLA-enriched bio-γ-linolenic acid from mold (BIO), SFO containing about 75% LA, palm oil (PLO) containing abundant saturated acid, and SBO containing 8% ALA after long-term (13-wk) feeding.

MATERIALS AND METHODS

Animals and diets. Male F344/DuCrj rats (8-wk-old) were purchased from Charles River Japan Inc. (Yokohama, Japan). Thirty-six animals were housed individually in cages in a room with controlled temperature (23 ± 1°C), humidity (60 ± 5%), and light (12-h light/dark cycle). The composition of the experimental diet was as follows (wt%): casein, 20; fat 10; cornstarch, 15; cellulose powder, 5; DL-methionine, 0.3; mineral mixture, 3.5 (AIN-76) (9); vitamin mixture, 1 (AIN-76) (9); choline bitartrate, 0.2; cholesterol, 0.5; sodium cholate, 0.125; and sucrose to 100. OBLO was extracted as described elsewhere (8). EPO, BIO, SFO, PLO, and SBO were purchased from Summit Oil Co. (Chiba, Japan, licensed by

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Abbreviations: AA 20:4n-6, arachidonic acid; ALA 18:3n-3, α-linolenic acid; BIO, bio-γ-linolenic acid from mold; DG, diacylglycerol; DHA 22:6n-3, docosahexaenoic acid; EPA 20:5n-3, eicosapentaenoic acid; EPO, evening primrose oil; GLC, gas–liquid chromatography; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LA 18:2n-6, linoleic acid; LDL, low density lipoprotein; OBLO, *Oenothera biennis* Linn oil; PC, phosphatidylcholine; PLO, palm oil; P/S, polyunsaturated fatty acid/saturated fatty acid; SBO, soybean oil; SFO, safflower oil; TG, triacylglycerol; VLDL, very low density lipoprotein.

Efamol Ltd.), Idemitsu Material Co. (Tokyo, Japan), Hounen Co. (Tokyo, Japan), Asahi Electric Industry Co. (Tokyo Japan), and Fuji Oil Co. (Osaka, Japan), respectively. The lipid class of each oil was as follows: OBLO was composed of 79% triacylglycerol (TG), 6% diacylglycerol (DG), 9% sterol, and other components; EPO was composed of 86% TG, 4% DG, 9% sterol, and other components; BIO was composed of 70% TG, 2% DG, 24% sterol, and other components; SFO was composed of 84% TG, 2% DG, 9% sterol, and other components; PLO was composed of 78% TG, 5% DG, 9% sterol, and other components; and SBO was composed of 80% TG, 1% DG, 13% sterol, and other components. Sitosterol comprised about 77–92% of the sterol concentration in each oil except for BIO, which contained 13% sitosterol and 57% ergosterol in the sterol concentration.

Table 1 shows the fatty acid compositions of the dietary fats. All rats (8-wk-old) were divided into six groups of six animals each. Rats were allowed free access to experimental diets and water for 13 wk. To avoid autooxidation, each diet was stored at −20°C and freshly prepared each day. All animal procedures described conformed to recognized principles (see Ref. 10).

Analytical procedures. Blood samples (2 mL) were collected between 0800 and 1000 h from the jugular veins of fed rats. The samples were taken into tubes without anticoagulant. After the samples stood at room temperature for 2 h, serum was prepared by centrifugation at 1500 × *g* for 20 min. At the end of the experimental period of 13 wk, all fecal excretion during 2 d was collected. Fecal dry weights did not differ among groups. The rats were killed by ether inhalation, and the livers quickly removed, washed with cold saline (9 g NaCl/L), blotted dry on filter paper, and weighed before freezing for storage.

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

Total lipids were extracted from serum, liver, and feces by a mixture of chloroform/methanol (2:1, vol/vol) (11). The serum and liver phosphatidylcholine (PC) were separated by two-step single-dimensional thin-layer chromatography on a silica gel plate; the solvents were chloroform/methanol (one-step; 95:5, vol/vol) and chloroform/acetone/methanol/acetic acid/water (two-step; 50:20:15:10:5, by vol). The fatty acids of the PC fraction were esterified in HCl-methanol (5 mL/L) for 2 h at 125°C (12) and analyzed using gas–liquid chromatography (GLC; Shimadzu 14A, Kyoto, Japan) with a 10% DEGS packed column (2 mm × 2.6 m, support shimalite w; Shimadzu) with nitrogen as the carrier gas. Neutral sterols in feces and liver obtained by saponification were acetylated (13) and analyzed by GLC with a DB 17 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA). Acidic sterols in feces were measured by GLC following the method of Grundy *et al*. (14).

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

RESULTS

Feed intake, rat growth, and liver weight. The results are summarized in Table 2. Dietary OBLO and BIO tended to decrease the body-weight gain significantly more than dietary EPO and SBO. The body weight of the dietary SFO group also increased significantly more than in the dietary OBLO group. There were no significant differences in the food intake among the groups. The liver weight in the OBLO group was the lowest of all groups, and that in the BIO group was significantly lower than in the PLO and EPO groups.

Tissue lipid concentration. The serum total, VLDL + IDL + LDL-cholesterol, and HDL-cholesterol concentrations are shown in Table 3. The total and VLDL + IDL + LDL-cholesterol concentrations in the OBLO group were significantly lower than those in the other groups, except for the EPO group, throughout the experimental period. Those in the EPO group were significantly lower than in the PLO and SBO groups throughout the experimental period. Those in the BIO and SBO groups were significantly lower than in the PLO group. Though the HDL-cholesterol concentration in the BIO

TABLE 1
Fatty Acid Composition of Dietary Fats

	Fatty acids (wt%)								
Dietary fats	14:0	16:0	16:1n-7	18:0	18:1n-9	18:2n-6	18:3n-3	18:3n-6	P/S[a]
Oenothera biennis Linn oil	—	7.2	—	1.4	6.2	71.0	—	13.7	11.7
Evening primrose oil	—	7.7	0.4	2.0	9.6	71.1	—	9.2	10.4
Bio-γ-linolenic acid oil	1.8	18.1	2.3	1.5	34.3	17.3	0.8	23.1	2.1
Safflower oil	0.1	8.2	—	2.3	13.0	75.2	0.5	—	9.1
Palm oil	1.3	46.4	—	3.5	37.9	10.1	0.4	—	0.2
Soybean oil	0.1	12.6	—	3.6	22.4	52.9	8.0	—	4.8

[a]Polyunsaturated/saturated ratio (P/S) = (18:2 + 18:3)/(14:0 + 16:0).

TABLE 2
Body Weight Gain, Food Intake, and Liver Weight in Rats Fed Dietary Fats (for 13 wk)[a]

Diet	Body weight		Food intake (g/13 wk)	Liver weight (wet g/100 g body weight)
	Initial (g)	Gain (g/13 wk)		
OBLO	185 ± 6	172 ± 11^{c}	1407 ± 53	4.0 ± 0.4^{d}
EPO	184 ± 4	202 ± 19^{a}	1474 ± 73	5.4 ± 0.2^{b}
BIO	185 ± 4	180 ± 9^{b,c}	1437 ± 62	4.9 ± 0.4^{c}
SFO	190 ± 5	197 ± 8^{a,b}	1473 ± 42	5.4 ± 0.4^{b,c}
PLO	184 ± 9	186 ± 18^{a,b,c}	1492 ± 139	6.1 ± 0.2^{a}
SBO	187 ± 8	203 ± 22^{a}	1500 ± 124	5.4 ± 0.7^{b,c}

[a]Values are expressed as means ± standard deviations for six rats. Means within the same columns bearing different superscripts are significantly different ($P < 0.05$). OBLO, *Oenothera biennis* Linn oil; EPO, evening primrose oil; BIO, bio-γ-linolenic acid oil; SFO, safflower oil; PLO, palm oil; SBO, soybean oil.

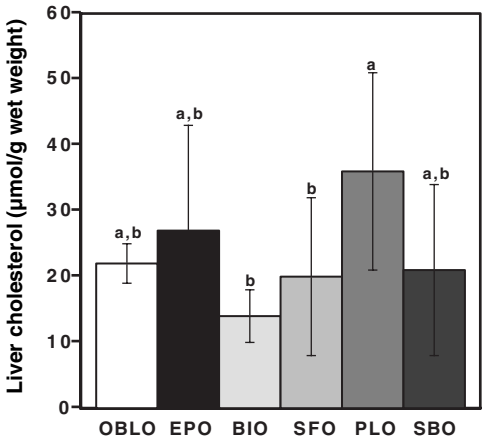

FIG. 1. Liver cholesterol concentration in rats fed dietary fats for 13 wk. Values are means for six rats, with standard deviations indicated by bars. Means values (a,b) were significantly different ($P < 0.05$); OBLO, *Oenothera biennis* Linn oil; EPO, evening primrose oil; BIO, bio-γ-linolenic acid oil; SFO, safflower oil; PLO, palm oil; SBO, soybean oil.

group was significantly higher than that in the other groups at 7 wk, the HDL-cholesterol concentration was significantly lower than in the other groups, except for the OBLO group, at the 13th wk.

Figure 1 illustrates the cholesterol concentrations in the livers of rats at the end of the 13-wk feeding period. Dietary PLO increased the liver cholesterol concentration significantly more than BIO and SFO did.

Fatty acid composition of the tissue. The fatty acid compositions of PC in the serum and liver are shown in Table 4. The level of 18:2n-6 in the serum of the BIO group increased significantly compared with the OBLO, EPO, and SFO groups. The level of 18:3n-6 in the OBLO group was significantly higher than in the other groups. The level of 20:4n-6 in the PLO group was significantly higher than that in the OBLO, EPO, and SBO groups.

The level of 18:2n-6 in the liver of the BIO group decreased significantly in all groups. The level of 18:3n-6 in the BIO group decreased significantly compared with the EPO group. The level of 20:4n-6 in the PLO group was significantly higher than in the other groups, except for the SBO group. The level of 22:6n-3 in the SBO group was significantly higher than in the other groups except for the OBLO group.

Fecal lipid concentration. Table 5 shows the effects of the dietary fat on fecal neutral sterol and bile acid concentrations in rats at the end of the experimental period. The excretion of cholesterol increased in the BIO group, and the excretion of coprostanol in the PLO group was significantly higher than in the OBLO group.

The excretion of chenodeoxycholic acid in the SFO group was highest among the groups, and the excretions of deoxycholic acid and lithocholic acid in the BIO group were significantly higher than those in the other groups. The total bile acid concentrations in the BIO and SFO groups also were significantly higher than in the other groups.

TABLE 3
Serum Total Cholesterol, VLDL + IDL + LDL-Cholesterol, and HDL-Cholesterol Concentrations in Rats Fed Dietary Fats (for 13 wk)[a]

Diet	0 Wk	3 Wk	7 Wk	13 Wk
Total cholesterol (mmol/L)				
OBLO	2.0 ± 0.1	2.7 ± 0.3^{d}	3.5 ± 0.4^{c}	4.3 ± 0.9^{d}
EPO	2.0 ± 0.2	3.2 ± 0.2^{c,d}	4.1 ± 0.6^{b,c}	4.9 ± 0.4^{c,d}
BIO	1.9 ± 0.1	4.1 ± 0.5^{b}	5.4 ± 0.7^{b}	6.5 ± 0.6^{b,c}
SFO	2.0 ± 0.2	3.5 ± 0.4^{b,c}	4.8 ± 0.9^{b,c}	6.3 ± 0.9^{b,c}
PLO	2.0 ± 0.2	5.0 ± 0.9^{a}	7.8 ± 3.0^{a}	9.4 ± 2.6^{a}
SBO	2.0 ± 0.3	3.9 ± 0.3^{b}	5.7 ± 1.3^{b}	7.0 ± 1.7^{b}
VLDL + IDL + LDL-cholesterol (mmol/L)				
OBLO	0.2 ± 0.1	1.0 ± 0.2^{d}	1.3 ± 0.2^{c}	2.1 ± 0.8^{c}
EPO	0.3 ± 0.1	1.5 ± 0.2^{c,d}	1.8 ± 0.4^{b,c}	2.3 ± 0.3^{c}
BIO	0.3 ± 0.1	2.0 ± 0.2^{b,c}	2.6 ± 0.8^{b,c}	4.6 ± 0.8^{b}
SFO	0.3 ± 0.1	1.9 ± 0.3^{b,c}	2.6 ± 0.8^{b,c}	4.0 ± 0.7^{b}
PLO	0.3 ± 0.1	3.6 ± 0.8^{a}	5.6 ± 2.9^{a}	6.9 ± 2.5^{a}
SBO	0.3 ± 0.1	2.4 ± 0.1^{b}	3.5 ± 1.3^{b}	4.7 ± 1.6^{b}
HDL-cholesterol (mmol/L)				
OBLO	1.7 ± 0.1	1.7 ± 0.1^{b}	2.2 ± 0.2^{b}	2.2 ± 0.2^{b,c}
EPO	1.7 ± 0.1	1.7 ± 0.1^{b}	2.3 ± 0.2^{b}	2.6 ± 0.2^{a}
BIO	1.6 ± 0.1	2.1 ± 0.4^{a}	2.8 ± 0.3^{a}	1.9 ± 0.3^{c}
SFO	1.7 ± 0.1	1.6 ± 0.1^{b,c}	2.2 ± 0.1^{b}	2.3 ± 0.2^{a,b}
PLO	1.7 ± 0.1	1.4 ± 0.2^{c}	2.2 ± 0.2^{b}	2.5 ± 0.2^{a,b}
SBO	1.7 ± 0.2	1.4 ± 0.2^{c}	2.2 ± 0.1^{b}	2.2 ± 0.1^{b}

[a]Values are expressed as means ± standard deviations for six rats. Means within the same columns bearing different superscripts are significantly different ($P < 0.05$). VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. See Table 2 for other abbreviations.

DISCUSSION

In a previous study (8), we reported the comparative hypocholesterolemic effects of six vegetable oils in cholesterol-fed rats after 6-wk feeding. The serum total and VLDL + IDL + LDL-cholesterol concentrations of 10 and 20% OBLO groups were consistently lower than those for other oils containing about 9% ALA, 40–64% LA or about 50% oleic acid, and for mixed oil prepared with an n-6/n-3 ratio of 3.5 and polyunsaturated fatty acid/saturated fatty acid (P/S) ratio of

TABLE 4
Fatty Acid Composition of Phosphatidylcholine in Serum and Livers of Rats Fed Dietary Fats (for 13 wk)[a]

	Diet					
Fatty acid	OBLO	EPO	BIO	SFO	PLO	SBO
Serum phosphatidylcholine						
14:0	12.2 ± 2.5[a]	10.9 ± 9.2[a,b]	3.1 ± 0.9[b,c]	7.5 ± 4.8[a,b,c]	2.0 ± 2.3[c]	5.2 ± 6.7[a,b,c]
16:0	47.0 ± 6.3[a]	40.2 ± 7.6[a,b]	32.9 ± 2.9[b,c]	38.2 ± 15.7[a,b]	28.6 ± 9.2[b,c]	23.3 ± 12.0[c]
16:1n-7	2.5 ± 1.0	2.7 ± 1.0	2.3 ± 2.7	12.2 ± 14.7	5.2 ± 7.2	4.4 ± 4.4
18:0	20.4 ± 4.7	22.8 ± 9.9	20.0 ± 8.3	15.7 ± 8.6	26.5 ± 12.3	19.0 ± 12.5
18:1n-9	13.5 ± 8.7[b]	12.1 ± 10.4[b]	26.1 ± 11.4[a,b]	16.6 ± 9.0[b]	19.1 ± 11.0[b]	38.2 ± 15.1[a]
18:2n-6	4.0 ± 1.3[c]	9.5 ± 5.7[b]	15.2 ± 2.4[a]	5.1 ± 3.4[b,c]	10.3 ± 5.1[a,b]	10.6 ± 5.9[a,b]
18:3n-6	1.5 ± 0.6[a]	0.5 ± 0.2[b]	0.2 ± 0.2[b]	<0.1	<0.1	<0.1
18:3n-3	1.7 ± 1.1	1.0 ± 0.5	0.7 ± 0.1	1.9 ± 1.7	0.7 ± 0.2	1.6 ± 1.3
20:4n-6	1.3 ± 0.6[b]	1.8 ± 2.3[b]	4.5 ± 3.4[a,b]	2.6 ± 1.6[a,b]	8.7 ± 6.5[a]	1.0 ± 0.8[b]
22:6n-3	<0.1	0.2 ± 0.2	0.1 ± 0.1	<0.1	0.6 ± 0.5	3.5 ± 2.9
Liver phosphatidylcholine						
14:0	2.1 ± 0.4[b]	5.7 ± 3.5[a]	1.2 ± 0.6[b]	2.2 ± 1.4[b]	3.0 ± 3.6[a,b]	5.8 ± 3.1[a]
16:0	21.9 ± 2.9[b]	25.2 ± 5.2[a,b]	22.4 ± 3.0[b]	28.0 ± 4.8[a,b]	27.5 ± 4.3[a,b]	30.4 ± 6.9[a]
16:1n-7	1.6 ± 1.4	2.0 ± 1.7	1.4 ± 1.3	5.2 ± 4.0	1.9 ± 2.1	1.9 ± 1.8
18:0	26.8 ± 7.1[a]	21.0 ± 3.1[a,b]	24.0 ± 1.9[a]	15.1 ± 4.8[b]	20.7 ± 7.6[a,b]	21.2 ± 6.7[a,b]
18:1n-9	13.7 ± 10.3[a,b]	12.1 ± 4.0[b]	9.4 ± 1.2[b]	22.1 ± 10.5[a]	19.0 ± 3.8[a,b]	19.0 ± 10.3[a,b]
18:2n-6	11.2 ± 1.5[a]	11.5 ± 2.6[a]	3.1 ± 0.9[b]	11.1 ± 2.4[a]	13.5 ± 8.9[a]	12.7 ± 4.4[a]
18:3n-6	0.4 ± 0.1[b]	0.3 ± 0.1[b]	1.0 ± 0.2[a]	<0.1	<0.1	<0.1
18:3n-3	0.5 ± 0.3[b]	0.4 ± 0.2[b]	0.2 ± 0.1[b]	0.5 ± 0.2[b]	1.2 ± 1.4[a]	0.8 ± 0.2[a,b]
20:4n-6	21.2 ± 7.1[b]	22.3 ± 6.5[b]	33.1 ± 1.5[a]	15.4 ± 5.7[b]	15.3 ± 6.2[b]	15.2 ± 7.4[b]
22:6n-3	1.0 ± 0.4[a,b]	0.5 ± 0.1[c,d]	0.8 ± 0.1[b,c]	0.6 ± 0.2[b,c,d]	0.3 ± 0.1[d]	1.3 ± 0.6[a]

[a]Values are expressed as means ± standard deviations of diets (wt%) for six rats. Means within the same rows bearing different superscripts are significantly different ($P < 0.05$). See Table 2 for abbreviations.

1.0 (8). Ishihara *et al*. (4) also suggested that n-6 fatty acid, LA, had no hypocholesterolemic effect in long-term feeding (120 d). In this long-term feeding experiment (13 wk), the OBLO from a wild plant was compared with EPO from a cultivated plant, BIO from mold, SFO containing about 75% LA, PLO containing about 46% palmitic acid, and SBO containing about 8% ALA and 53% LA.

The body-weight gain was less in the OBLO and BIO groups than in EPO and SBO groups, and the liver weight was less in the OBLO and BIO groups than in the other groups, although food intake was similar among the six groups. The data for body weight in the dietary OBLO group agreed with previous studies (15–17). Takada *et al*. (15) reported that dietary GLA oil reduced body-fat content and facilitated fatty acid β-oxidation in the liver, and the reduction of body weight in this experiment might have been due to those effects.

This study indicated that dietary OBLO with an average level of 212 mg of 18:3n-6/d was more efficient in lowering serum total cholesterol than SFO, PLO, or SBO, which contained abundant LA, saturated fatty acid, and LA + ALA, respectively, without GLA, and that dietary EPO at an average level of 149 mg of 18:3n-6/d also was more efficient than PLO or SBO in long-term feeding. The results for the dietary OBLO and EPO groups agreed with a report on rats fed at levels of about 130 and 177 mg of 18:3n-6/d (18). Ishihara *et al*. (4) reported that increased intake of high-linoleate vegetable oil was not useful for the prevention of hypercholesterolemia-associated diseases; however, dietary oils rich in n-3 fatty

TABLE 5
Fecal Steroid Concentrations in Rats Fed Dietary Fats (for 13 wk)[a]

	Diet					
Component	OBLO	EPO	BIO	SFO	PLO	SBO
Cholesterol	15.2 ± 5.2[b]	16.3 ± 7.3[a,b]	22.7 ± 8.7[a]	10.0 ± 2.4[b]	14.4 ± 2.7[b]	14.9 ± 4.0[b]
Coprostanol	1.26 ± 0.64[b]	1.84 ± 1.48[a,b]	2.90 ± 1.41[a,b]	1.76 ± 1.00[a,b]	3.35 ± 1.77[a]	2.19 ± 1.74[a,b]
LCA	1.20 ± 0.43[c]	0.92 ± 0.15[c]	3.86 ± 0.86[a]	2.08 ± 0.59[b]	1.03 ± 0.36[c]	1.20 ± 0.94[c]
DCA	0.07 ± 0.03[b]	0.05 ± 0.01[b]	0.56 ± 0.16[a]	0.10 ± 0.03[b]	0.13 ± 0.14[b]	0.11 ± 0.06[b]
CDCA	0.12 ± 0.04[b]	0.06 ± 0.01[b,c]	0.13 ± 0.14[b]	0.23 ± 0.07[a]	0.01 ± 0.01[c]	0.03 ± 0.02[c]
CA	1.85 ± 0.99[c]	1.57 ± 0.66[c]	4.93 ± 2.74[a,b]	5.46 ± 2.15[a]	3.00 ± 1.63[b,c]	3.45 ± 1.49[a,b,c]
TBA	3.20 ± 1.46[b]	2.60 ± 0.79[b]	9.46 ± 3.75[a]	7.88 ± 2.60[a]	4.17 ± 2.04[b]	3.93 ± 1.68[b]

[a]Values are expressed as means ± standard deviations of diets (μmol/100 g body weight/d) for six rats. Means within the same rows bearing different superscripts are significantly different ($P < 0.05$). Abbreviations: LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; TBA, total bile acid. See Table 2 for other abbreviations.

acids might be useful. Although dietary GLA is hypocholesterolemic compared with SBO containing 8% ALA (8), our experiments did not include perilla oil, which contains abundant ALA, and we did not study aged rats. Thus, our results might not agree with results of Ishihara *et al*. (4) because our conditions were different from theirs (4). However, Horrobin and Manku (19) have reported that GLA, the first essential fatty acid metabolite of LA, has cholesterol-lowering action greater than that of the parent molecule, suggesting that LA must be converted to GLA to exert its desirable effects on cholesterol metabolism. They also indicated that the cholesterol-lowering effect of LA is probably not due to LA itself but to that fraction which is converted to GLA and beyond and, in patients with elevated cholesterol levels, the Δ6-desaturase enzyme that converts LA to GLA appears to be functioning inefficiently. Their speculations agreed with our results, and diets containing GLA had a greater hypocholesterolemic effect than diets without GLA.

The cholesterol content in the livers of rats fed the BIO diet was the lowest among the six groups. Consistent with this finding were the data which indicated that total bile acid and cholesterol levels in feces were the highest among the six groups. Dietary BIO at an average level of 365 mg of 18:3n-6/d was shown to be efficient in inhibition against increasing the serum total cholesterol level compared with dietary PLO. The hypocholesterolemic effect depends on the P/S ratio of dietary fats, and Lee *et al*. (20) reported that the P/S ratio (11.1) of SFO was more effective than the P/S ratios (0.2 and 1.8) of PLO and SFO + PLO. Thus, it may be the total amount of polyunsaturates that is important and not one single fatty acid, and the inhibition effect against increasing serum cholesterol concentration of their polyunsaturated oils is not due to increased fecal excretion of neutral and acidic sterols.

The level of 18:3n-6 in the serum and liver PC was detected in OBLO, EPO, and BIO groups. The result shows that dietary GLA affected the amount of 18:3n-6 in serum and liver PC.

The dietary OBLO and EPO could inhibit the increasing of serum VLDL + IDL + LDL-cholesterol concentrations in rats throughout the experimental period compared with the other oils. Grundy and Denke (21), in a review, reported that the n-6 fatty acid replacing saturated acids in the diet reduces the number of LDL particles in circulation, as revealed by a definite fall in LDL-apoB concentrations, inhibits hepatic synthesis of apoB-containing lipoproteins, and increases LDL-receptor activity. The data in this experiment may reflect such mechanisms, because the data for the liver cholesterol concentrations and fecal sterol concentrations in the OBLO and EPO groups alone could not explain the inhibition effects against the increasing of serum total cholesterol and VLDL + IDL + LDL-cholesterol concentrations.

In conclusion, the results of this study demonstrate that OBLO from a wild plant and EPO from a cultivated plant inhibit the increasing of serum total cholesterol and VLDL + IDL + LDL-cholesterol concentrations as compared with the BIO, SFO, PLO, and SBO in the presence of excess cholesterol in the diet after long-term feeding.

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Low-Dose Eicosapentaenoic or Docosahexaenoic Acid Administration Modifies Fatty Acid Composition and Does Not Affect Susceptibility to Oxidative Stress in Rat Erythrocytes and Tissues

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ABSTRACT: In view of the promising future for use of n-3 polyunsaturated fatty acids (PUFA) in the prevention of cancer and cardiovascular diseases, it is necessary to ensure that their consumption does not result in detrimental oxidative effects. The aim of the present work was to test a hypothesis that low doses of eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) do not induce harmful modifications of oxidative cell metabolism, as modifications of membrane fatty acid composition occur. Wistar rats received by gavage oleic acid, EPA, or DHA (360 mg/kg body weight/day) for a period of 1 or 4 wk. Fatty acid composition and α-tocopherol content were determined for plasma, red blood cell (RBC) membranes, and liver, kidney, lung, and heart microsomal membranes. Susceptibility to oxidative stress induced by *tert*-butylhydroperoxide was measured in RBC. EPA treatment increased EPA and docosapentaenoic acid (DPA) content in plasma and in all the membranes studied. DHA treatment mainly increased DHA content. Both treatments decreased arachidonic acid content and n-6/n-3 PUFA ratio in the membranes, without modifying the Unsaturation Index. No changes in tissue α-tocopherol content and in RBC susceptibility to oxidative stress were induced by either EPA or DHA treatment. The data suggest that EPA and DHA treatments can substantially modify membrane fatty acids, without increasing susceptibility to oxidative stress, when administered at low doses. This opens the possibility for use of low doses of n-3 PUFA for chemoprevention without risk of detrimental secondary effects.
Lipids 32, 1075–1083 (1997).

As a part of a recent study of the biological activity of n-3 polyunsaturated fatty acids (PUFA), we found that dietary supplementation with mixtures of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in human subjects at high risk for colon cancer, suppressed cytokinetic anomalies in the colonic mucosa (1,2). This indicates that these fatty acids may be protective against cancer and supports our earlier observations and those of others in animal models (3–6). In view of the promising future use for n-3 PUFA in the prevention of cancer and cardiovascular diseases (5,7–9), it is necessary to ensure that their consumption does not result in detrimental side effects. The substitution of n-6 PUFA with potentially unstable n-3 PUFA in the membranes may shift fatty acid composition toward a higher degree of unsaturation and enhance membrane susceptibility to lipid peroxidation (10,11). Moreover, increased uptake of PUFA may affect vitamin E status either by impairing the absorption of the antioxidant (12,13) or by causing increased vitamin E consumption in plasma and tissues owing to enhanced lipid peroxidation (14,15).

We reported earlier that treatment of human subjects with n-3 PUFA at low doses and without significant addition of α-tocopherol did not produce any detrimental effect, whereas high doses resulted in harmful modifications of oxidative metabolism, such as an increase of red blood cell (RBC) susceptibility to lipid peroxidation and changes in endogenous tocopherol content and Mg-K-ATPase activity (16,17).

The aim of the present study was to evaluate in a rat model short- and longer-term effects of low dietary administrations of EPA or DHA without significant supplementation of vitamin E on membrane fatty acid composition of a variety of tissues, on RBC susceptibility to lipid peroxidation, and on tissue α-tocopherol content. n-3 Fatty acids were given separately and at doses comparable to the minimum used in our human studies (1,2,16,17).

MATERIALS AND METHODS

Animals. Thirty male Wistar rats (~250 g) were fed a nonpurified commercial diet (Altromin-Rieper, Rieper Company, Bolzano, Italy). The composition of the diet was: (%, w/w) crude protein, 23; fat, 5.5; fiber, 5; mineral, 8; carbohydrate, 58.5; water, 12. The vitamin mixture added to the diet was 2.5 g/kg (vitamin E, 100 mg/kg), and the mineral mixture was

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Abbreviations: DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; OA, oleic acid; PUFA, polyunsaturated fatty acids; RBC, red blood cell; *t*-BOOH, *tert*-butylhydroperoxide.

0.52 g/kg by weight. The diet provided 7.5 g/kg n-6 PUFA as linoleic acid (18:2n-6) and 1.02 g/kg n-3 PUFA as 18:3, 18:4, 20:5 and 22:6 . The dietary n-3/n-6 PUFA ratio was 0.14 and increased to 1.20 after EPA or DHA administration. Food and water were provided *ad libitum*.

The animals were randomly divided into three groups of 10 animals each. One group received oleic acid ethyl ester (OA, control), a second group received EPA-ethyl ester, and a third DHA-ethyl ester. The oils were given by gastric gavage as a single dose of 360 mg/kg body weight/day. The amount of OA (18:1n-9) in the control group was adjusted to equalize caloric intake among the groups. Preliminary experiments comparing OA-treated animals with untreated animals showed that OA treatment did not modify fatty acid composition and α-tocopherol content in plasma and cell membrane fractions.

The dietary dose (360 mg/kg body weight) was chosen to approximate the daily oral dose (2.5 g) that was found to normalize altered cellular proliferation in the colonic mucosa of patients at high risk for colon cancer (1,2). The same dose was found not to produce any adverse effects (16,17). Body surface area was used as the reference for conversion of the human dosage to the rat (18). An n-3 PUFA dose of 2.5 g/d given to patients weighing about 70 kg corresponded to a daily intake of 300 mg/kg body weight in the rat. In the human study, the n-3 PUFA were taken in three daily subdoses to facilitate absorption from the intestine. We increased the dose to 360 mg/kg body weight/d to compensate for reduced absorption when single doses of n-3 PUFA were used in the rat model.

The α-tocopherol amount (3 mg/g) was added to the oils for prevention of autooxidation. Total intake of α-tocopherol was 1.77 mg/d, (1.5 mg/d from the diet plus 0.27 mg/d from oil supplementation). This approximated the intake in earlier human studies (1,2). The α-tocopherol daily intake during the human studies was the sum of the content in the standard Italian diet (13 mg/d) plus the content in oil capsules (0.9–2.7 mg/d) and was similar to the recommended dietary intake (19,20).

The purity of fatty acids was 92 and 90% for EPA and DHA, respectively. Their α-tocopherol content was verified by high-performance liquid chromatography.

Food consumption and body weight gain for the animals were measured twice a week. Five animals from each group were sacrificed after 1 wk of treatment and the remaining five animals after 4 wk. The animal use protocol was approved by the Ministry of Health, Veterinary Service, Rome, Italy.

Sample collection. The animals were decapitated after ether anesthesia, and the blood was collected in heparinized tubes. Plasma was separated by centrifugation at $1000 \times g$ for 10 min at 4°C. RBC were washed three times in buffered isotonic NaCl solution. RBC membranes were prepared as described by Burton *et al.* (21). Liver, kidneys, lungs, and heart were rapidly excised and immediately frozen in liquid N_2 and stored at −80°C prior to processing. Microsomal membranes were prepared from liver, kidneys, heart, and lungs as described by Bartoli *et al.* (22) and protein concentration was measured by the Bradford assay (23).

Analysis of fatty acids. Lipids were extracted from microsomal membranes by the method of Bligh and Dyer (24), using chloroform/methanol (2:1, vol/vol) containing butylated hydroxytoluene (0.01%), and gravimetrically measured (25). Total phospholipids were separated from neutral lipids by thin-layer chromatography using silica gel 60 plates (Merck, Darmstadt, Germany) and toluene/diethyl ether/ethanol 35:3.5:1 (by vol) as a solvent system. Fatty acids in the phospholipid band were transesterified with the use of methanolic-HCl (3 N) (Supelco, Bellefonte, PA) at 70°C for 2 h. After extraction with *n*-hexane, methyl esters were separated and quantified by means of an HRGC 5300 Megaseries gas–liquid chromatograph (Carlo Erba, Milan, Italy) equipped with a 30 m × 0.25 mm capillary column (Supelco, Omegawax 320) using helium as carrier gas. The oven temperature was programmed at 1°C/min from 180 to 220°C. Retention times were identified with standards (Sigma Chemical Co., St. Louis, MO). Peaks corresponding to 16:0, 18:0, 18:1n-9, 18:2n-6, 18:3n-6, 18:3n-3, 20:3n-6, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-3, and 22:6n:3 were detected by the system and their areas measured with a Carlo Erba Megaseries integrator. Unsaturation Index was measured as the sum of the percentages of individual fatty acids × number of double bonds.

Oxidative stress induced by tert-*butylhydroperoxide (t-BOOH) in RBC.* A 5% RBC suspension in phosphate-buffered saline (pH 7.4) was incubated with 0.5 mM *t*-BOOH at 37°C under oxygen for 90 min. At each time point, a sample of 1 mL was taken for analysis of both malondialdehyde formation at 533 nm (26) and K^+ efflux. K^+ efflux was determined by flame emission photometry and expressed as percentage of total efflux evoked by lysis of RBC in distilled water (27).

Vitamin E analysis. α-Tocopherol was extracted in hexane from plasma, RBC membranes, and tissue microsomal membranes using, respectively, 25 µL plasma and a membrane equivalent of 7 mg protein. Samples were dissolved in methanol, and 20 µL aliquots were analyzed by high-performance liquid chromatography fluorescence detection on a Perkin-Elmer (Norwalk, CT) 650-LC fluorescence detector at 295 nm excitation, 340 nm emission. α-Tocopherol, as well as the internal standard, tocol, was eluted with 100% methanol on Alltech (Deerfield, IL) C-183-µm column, as indicated in Reference 28.

Statistical analysis. The results are expressed as means ± SE, and significance was assessed by one-way analysis of variance using Minitab software (Minitab Inc., State College, PA). When significant differences were found, *post hoc* comparisons of means were made using Fisher's test. Differences were considered significant at $P < 0.05$.

RESULTS

Food intake and body weight gain were similar in all groups throughout the 4-wk feeding period. Treatments did not modify the content of total lipids in the tissues, as shown by lipid/protein ratios of 0.20 ± 0.01, 1.16 ± 0.11, 0.77 ± 0.04, 0.65 ± 0.05, 1.07 ± 0.12, and 1.39 ± 0.09 in plasma, RBC,

TABLE 1
Fatty Acid Composition (% of total) of Plasma Phospholipids in Rats Treated with Oleic Acid (OA), Eicosapentaenoic Acid (EPA), and Docosahexaenoic Acid (DHA) (for 1 and 4 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Saturated	32.99 ± 0.41	33.01 ± 1.48	33.24 ± 1.15	34.22 ± 0.95	34.52 ± 1.13	35.00 ± 2.46
Monounsaturated	15.61 ± 1.08	14.05 ± 1.27	17.23 ± 0.67	15.91 ± 1.29	15.85 ± 1.30	15.96 ± 0.78
18:2n-6	26.12 ± 2.01	27.02 ± 2.32	29.28 ± 1.08	30.22 ± 0.81	27.56 ± 1.79	29.80 ± 1.34
18:3n-6	0.27 ± 0.03	0.32 ± 0.02	0.25 ± 0.03	0.29 ± 0.02	0.29 ± 0.06	0.24 ± 0.03
18:3n-3	0.71 ± 0.07	0.67 ± 0.04	1.15 ± 0.09[a]	1.18 ± 0.12[a]	0.87 ± 0.11	1.24 ± 0.06[a]
20:4n-6	14.99 ± 1.66	14.41 ± 1.36	9.60 ± 1.19[a]	11.12 ± 0.90[a]	11.53 ± 0.71[a]	11.09 ± 1.00[a]
20:5n-3	0.91 ± 0.13	0.75 ± 0.04	1.85 ± 0.18[a,b]	1.36 ± 0.13[a,b,c]	1.09 ± 0.06	0.99 ± 0.09[a]
22:4n-6	0.41 ± 0.07	0.54 ± 0.07	0.17 ± 0.10[a]	0.26 ± 0.02[a]	0.18 ± 0.03[a]	0.26 ± 0.05[a]
22:5n-3	0.81 ± 0.05	0.85 ± 0.05	1.22 ± 0.09[a]	1.20 ± 0.15[a,b]	0.53 ± 0.06[a]	0.76 ± 0.13
22:6n-3	3.32 ± 0.15	3.21 ± 0.48	2.57 ± 0.11[a]	2.78 ± 0.38	4.06 ± 0.25[a]	3.70 ±.0.56
Total n-6 PUFA	41.84 ± 1.55	42.58 ± 1.49	39.55 ± 1.84	41.68 ± 0.45	39.32 ± 1.93	41.17 ± 2.02
Total n-3 PUFA	6.30 ± 0.24	6.04 ± 0.52	7.22 ± 0.22[a]	6.33 ± 0.77	6.87 ± 0.46	6.78 ± 0.75
n-6/n-3 PUFA ratio	7.08 ± 0.54	8.23 ± 0.71	6.14 ± 0.25[a]	6.64 ± 0.33[a]	6.51 ± 0.37[a]	7.09 ± 0.82
Unsaturation Index	163.54 ± 7.03	162.48 ± 6.59	151.72 ± 6.05	154.87 ± 4.20	154.74 ± 6.07	156.39 ± 8.78

[a]$P < 0.05$ vs. OA group, same week.
[b]$P < 0.05$ for difference between EPA and DHA group, same week.
[c]$P < 0.05$ vs. first week. Abbreviation: PUFA, polyunsaturated fatty acid. All values are means ± SE of five determinations.

liver, kidney, heart, and lung membranes, respectively. These ratios did not change throughout short (1 wk) or long (4 wk) periods of treatment.

Tables 1–6 show fatty acid composition for total phospholipids extracted from plasma, RBC membranes, liver, kidney, heart, and lung microsomal membranes isolated from rats treated for 1 and 4 wk with OA, EPA, or DHA.

The content of saturated and monounsaturated fatty acids in plasma, RBC, and tissue membranes was unaffected by dietary supplementation with EPA and DHA. On the other hand, the content of total and individual PUFA changed significantly in rats treated with the two n-3 fatty acids as compared to the control group. Alterations in the PUFA pattern became evident within 7 d of treatment. EPA (20:5n-3) was incorporated to different extents in the tissues studied. Its increase in RBC membranes after 1 wk of EPA treatment was the highest (340%), followed by that observed in heart (218%), lung (157%), liver (107%), plasma (103%), and kid-

TABLE 2
Phospholipid Fatty Acid Composition (% of total) of Red Blood Cell Membranes Isolated from Rats Treated with OA, EPA, and DHA (for 1 and 2 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Saturated	43.12 ± 1.43	45.21 ± 2.27	44.23 ± 1.56	45.20 ± 1.89	42.64 ± 1.32	43.71 ± 1.60
Monounsaturated	11.26 ± 0.35	10.43 ± 0.56	10.40 ± 0.33	10.71 ± 0.46	9.74 ± 0.51	9.76 ± 0.40
18:2n-6	10.33 ± 0.47	10.88 ± 1.03	11.20 ± 0.48	11.25 ± 0.35	10.53 ± 0.45	12.02 ± 0.68
18:3n-6	0.09 ± 0.01	0.12 ± 0.02	0.11 ± 0.01	0.14 ± 0.02	0.08 ± 0.01	0.10 ± 0.02
18:3n-3	0.11 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
20:3n-6	0.51 ± 0.05	0.57 ± 0.08	0.45 ± 0.06	0.55 ± 0.08	0.60 ± 0.07	0.60 ± 0.03
20:4n-6	23.27 ± 0.67	22.88 ± 0.94	21.59 ± 0.63	21.15 ± 0.84	22.18 ± 0.53	21.90 ± 0.25
20:5n-3	0.40 ± 0.08	0.44 ± 0.06	1.76 ± 0.16[a,b]	1.52 ± 0.31[a,b]	0.55 ± 0.05[a]	0.58 ± 0.06[a]
22:4n-6	1.82 ± 0.09	1.55 ± 0.06	1.58 ± 0.09[a]	1.05 ± 0.16[a,c]	1.42 ± 0.07[a]	1.06 ± 0.08[a,c]
22:5n-3	2.22 ± 0.16	1.88 ± 0.14	2.90 ± 0.17[a,b]	3.28 ± 0.37[a,b]	1.83 ± 0.02	1.89 ± 0.18
22:6n-3	3.60 ± 0.22	3.20 ± 0.18	3.24 ± 0.10[b]	3.22 ± 0.45[b]	5.22 ± 0.50[a]	5.17 ± 0.43[a]
Total n-6 PUFA	36.56 ± 0.31	36.03 ± 1.44	35.05 ± 0.88	33.90 ± 0.84	35.84 ± 0.82	35.90 ± 0.56
Total n-3 PUFA	6.65 ± 0.20	5.76 ± 0.36[c]	7.56 ± 0.53[a]	8.15 ± 0.80[a]	8.16 ± 0.47[a]	8.01 ± 0.66[a]
n-6/n-3 PUFA ratio	5.52 ± 0.15	6.35 ± 0.52[c]	4.79 ± 0.45[a]	4.35 ± 0.49[a]	4.46 ± 0.25[a]	4.64 ± 0.46[a]
Unsaturation Index	170.61 ± 2.57	162.90 ± 5.57	168.13 ± 6.48	166.21 ± 4.87	179.86 ± 5.18	173.29 ± 4.50

[a]$P < 0.05$ vs. OA group, same week.
[b]$P < 0.05$ for difference between EPA and DHA group, same week.
[c]$P < 0.05$ vs. first week. See Table 1 for abbreviations. All values are means ± SE of five determinations.

TABLE 3
Phospholipid Fatty Acid Composition (% of total) of Liver Microsomal Membranes Isolated from Rats Treated with OA, EPA, and DHA (for 1 and 4 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Saturated	41.79 ± 1.17	41.04 ± 1.82	39.82 ± 1.33	39.62 ± 1.64	40.56 ± 2.00	41.20 ± 2.36
Monounsaturated	8.33 ± 0.18	7.62 ± 0.59	8.66 ± 0.49	7.48 ± 0.51	8.03 ± 0.67	7.78 ± 0.35
18:2n-6	15.56 ± 1.82	17.07 ± 0.77	16.14 ± 0.95	19.76 ± 0.90[a,c]	16.63 ± 0.21	19.66 ± 1.33[a,c]
18:3n-6	0.19 ± 0.03	0.19 ± 0.04	0.22 ± 0.04	0.18 ± 0.04	0.27 ± 0.03[a]	0.17 ± 0.04
18:3n-3	0.22 ± 0.05	0.25 ± 0.03	0.21 ± 0.02	0.36 ± 0.08	0.29 ± 0.04	0.24 ± 0.03
20:3n-6	0.75 ± 0.12	0.92 ± 0.03	1.01 ± 0.05[a]	1.07 ± 0.02[a]	1.16 ± 0.08[a]	1.32 ± 0.17[a]
20:4n-6	21.27 ± 0.69	23.39 ± 1.43	20.76 ± 0.88	20.80 ± 0.95[a]	18.52 ± 0.73[a]	19.06 ± 1.36[a]
20:5n-3	0.41 ± 0.11	0.55 ± 0.05	0.85 ± 0.11[a]	0.84 ± 0.11[a]	0.74 ± 0.04[a]	0.73 ± 0.07[a]
22:4n-6	0.38 ± 0.08	0.33 ± 0.03	0.28 ± 0.05	0.26 ± 0.03	0.24 ± 0.02[a]	0.21 ± 0.03[a]
22:5n-3	0.80 ± 0.05	0.81 ± 0.10	2.87 ± 0.39[a,b]	1.66 ± 0.26[a,b,c]	1.12 ± 0.09[a]	1.16 ± 0.10[a]
22:6n-3	5.58 ± 0.65	4.80 ± 0.57	6.29 ± 0.69[b]	5.48 ± 0.53[b]	10.40 ± 1.09[a]	8.90 ± 0.90[a,c]
Total n-6 PUFA	37.97 ± 1.50	41.20 ± 0.62[c]	37.95 ± 1.91	41.46 ± 0.34[c]	36.86 ± 0.891	40.32 ± 0.53[c]
Total n-3 PUFA	8.18 ± 1.53	6.39 ± 0.74	10.13 ± 1.05[b]	8.72 ± 0.92[a]	12.75 ± 0.83[a]	10.44 ± 0.41[a,c]
n-6/n-3 PUFA ratio	5.15 ± 1.63	7.05 ± 0.99	4.21 ± 0.60[a]	5.26 ± 0.76	3.10 ± 0.28[a]	4.39 ± 0.22[a]
Unsaturation Index	179.14 ± 11.18	176.26 ± 7.57	185.66 ± 3.57	181.76 ± 7.65	194.20 ± 4.24	176.79 ± 11.23

[a]$P < 0.05$ vs. OA group, same week.
[b]$P < 0.05$ for difference between EPA and DHA group, same week.
[c]$P < 0.05$ vs. first week. For abbreviations, see Table 1. All values are means ± SE of five determinations.

ney (87%). After 4 wk of EPA treatment, the level of this fatty acid decreased in plasma and heart membranes (Tables 1 and 5). Nevertheless, EPA was still higher than that observed in the control group. In lung (Table 6) it decreased to the control value, whereas in RBC, liver, and kidney membranes it remained relatively unchanged as compared to that observed after 1 wk (Tables 2–4).

EPA treatment did not modify the DHA content in plasma, RBC, and tissue membranes, whereas it significantly increased docosapentaenoic acid (DPA, 22:5n-3), which is a product of EPA elongation. This effect was exaggerated in heart and liver (259 and 127% increase, respectively). DHA did not modify DPA level in any of the tissues except liver, in which a slight (40%) but significant increase was observed (Table 3).

As observed for EPA, but to a lesser extent, increased incorporation of DHA (22:6n-3) occurred in plasma and tissue membranes following dietary supplementation. The increase in DHA was evident after 1 wk and with the exception of

TABLE 4
Phospholipid Fatty Acid Composition (% of total) of Kidney Microsomal Membranes Isolated from Rats Treated with OA, EPA, and DHA (for 1 and 4 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Saturated	41.90 ± 1.16	41.39 ± 0.10	41.06 ± 0.26	41.61 ± 1.67	42.85 ± 1.46	42.21 ± 1.88
Monounsaturated	10.20 ± 0.45	9.92 ± 0.44	9.69 ± 0.31	10.01 ± 0.72	10.09 ± 0.40	10.62 ± 0.87
18:2n-6	14.06 ± 0.90	14.79 ± 0.98	14.16 ± 0.73[b]	17.06 ± 0.58[a,c]	17.11 ± 0.94[a]	16.79 ± 0.68[a]
18:3n-6	0.18 ± 0.01	0.18 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.18 ± 0.10	0.18 ± 0.01
18:3n-3	0.15 ± 0.02	0.21 ± 0.04	0.13 ± 0.06	0.19 ± 0.04	0.19 ± 0.03	0.19 ± 0.03
20:3n-6	1.23 ± 0.03	1.16 ± 0.06	1.05 ± 0.08[a,b]	1.25 ± 0.11	1.35 ± 0.09	1.22 ± 0.02
20:4n-6	26.31 ± 1.08	27.76 ± 0.64	25.12 ± 1.66	23.23 ± 1.30[a]	23.29 ± 1.36[a]	24.12 ± 0.84[a]
20:5n-3	0.52 ± 0.04	0.48 ± 0.08	0.97 ± 0.15[a]	1.24 ± 0.12[a]	1.00 ± 0.21[a]	1.01 ± 0.15[a]
22:4n-6	0.58 ± 0.02	0.52 ± 0.01	0.47 ± 0.17	0.30 ± 0.02[a]	0.37 ± 0.02[a]	0.30 ± 0.04[a]
22:5n-3	0.34 ± 0.03	0.28 ± 0.02	0.58 ± 0.07[a,b]	0.55 ± 0.04[a,b]	0.31 ± 0.02	0.37 ± 0.05
22:6n-3	1.97 ± 0.18	2.12 ± 0.08	2.13 ± 0.13[b]	2.09 ± 0.19[b]	2.50 ± 0.24[a]	2.78 ± 0.37[a]
Total n-6 PUFA	42.28 ± 0.91	44.32 ± 1.32	40.44 ± 1.64	42.31 ± 1.20	42.37 ± 1.66	42.60 ± 1.45
Total n-3 PUFA	2.99 ± 0.06	3.07 ± 0.16	3.43 ± 0.53	4.07 ± 0.22[a]	4.00 ± 0.46[a]	4.36 ± 0.33[a]
n-6/n-3 PUFA ratio	15.91 ± 0.41	16.63 ± 0.38	13.70 ± 1.91	11.79 ± 0.53[a]	12.71 ± 1.54[a]	11.52 ± 0.61[a]
Unsaturation Index	166.36 ± 0.57	173.70 ± 3.40	160.27 ± 4.88	164.63 ± 5.27	165.90 ± 2.66	170.31 ± 3.93

[a]$P < 0.05$ vs. OA group, same week.
[b]$P < 0.05$ for difference between EPA and DHA group, same week.
[c]$P < 0.05$ vs. first week. For abbreviations see Table 1. All values are means ± SE of five determinations.

TABLE 5
Phospholipid Fatty Acid Composition (% of total) of Heart Microsomal Membranes Isolated from Rats Treated with OA, EPA, and DHA (for 1 and 4 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Saturated	34.96 ± 3.07	34.45 ± 1.10	34.51 ± 1.42	35.49 ± 2.07	35.58 ± 1.91	35.20 ± 1.6
Monounsaturated	8.88 ± 1.89	8.42 ± 0.49	9.76 ± 0.74	8.49 ± 0.61	9.82 ± 0.78	8.16 ± 0.54
18:2n-6	18.86 ± 1.97	20.83 ± 1.94	21.70 ± 1.86	24.64 ± 2.28	20.52 ± 2.13	23.44 ± 2.19
18:3n-6	0.13 ± 0.00	0.14 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.19 ± 0.03
18:3n-3	0.38 ± 0.09	0.30 ± 0.03	0.35 ± 0.07	0.28 ± 0.03	0.38 ± 0.04	0.29 ± 0.03
20:3n-6	0.52 ± 0.06	0.42 ± 0.04	0.47 ± 0.02	0.39 ± 0.02	0.51 ± 0.03	0.46 ± 0.03
20:4n-6	16.86 ± 0.45	17.57 ± 0.28	15.42 ± 1.00	15.17 ± 0.23[a,b]	14.85 ± 0.44[a]	14.22 ± 0.38[a]
20:5n-3	0.33 ± 0.10	0.23 ± 0.05	1.05 ± 0.09[a,b]	0.55 ± 0.04[a,b,c]	0.33 ± 0.07	0.21 ± 0.08
22:4n-6	1.07 ± 0.15	1.00 ± 0.17	0.91 ± 0.20	0.48 ± 0.02[a,c]	0.92 ± 0.18	0.45 ± 0.06[a,c]
22:5n-3	1.90 ± 0.27	1.73 ± 0.02	4.32 ± 0.09[a,b]	3.85 ± 0.39[a,b]	1.47 ± 0.29	1.40 ± 0.10
22:6n-3	8.13 ± 0.82	9.63 ± 0.71	8.87 ± 0.31[b]	10.15 ± 0.84[b,c]	15.19 ± 1.05[a]	14.56 ± 0.66[a]
Total n-6 PUFA	38.48 ± 2.17	41.45 ± 1.85	38.92 ± 2.13	39.47 ± 2.15	36.60 ± 2.12	38.68 ± 1.80
Total n-3 PUFA	10.85 ± 0.97	12.03 ± 1.17	12.33 ± 1.54[b]	14.39 ± 1.20[b]	17.45 ± 1.02[a]	16.55 ± 0.80[a]
n-6/n-3 PUFA ratio	3.67 ± 0.51	3.30 ± 0.45	3.41 ± 0.66[b]	2.83 ± 0.31	2.12 ± 0.24[a]	2.36 ± 0.20[a]
Unsaturation Index	189.14 ± 5.51	198.94 ± 6.63	190.23 ± 6.77[b]	198.76 ± 5.07	214.44 ± 6.03[a]	211.68 ± 5.96

[a] $P < 0.05$ vs. OA group, same week.
[b] $P < 0.05$ for difference between EPA and DHA group, same week.
[c] $P < 0.05$ vs. first week. For abbreviations, see Table 1. All values are means ± SE of five determinations.

plasma (Table 1), it was also present after 4 wk of treatment. During DHA supplementation the levels of EPA were elevated in plasma, RBC, liver, and kidney but remained unmodified in heart and lung.

The treatments with EPA or DHA were associated with modifications in n-6 PUFA content. In particular, linoleic acid (18:2n-6) was increased in plasma, heart, liver, and kidney (Tables 3 and 4). Nevertheless the change was significant only in liver and kidney. Similarly, a significant reduction in arachidonic acid (20:4n-6) was observed in plasma, liver, kidney, and heart after both EPA and DHA treatments, while no changes were found in RBC and lung.

Fatty acid modification was associated with a significant decrease in the n-6/n-3 PUFA ratio in plasma and in all the tissues examined from both EPA and DHA groups. On the contrary, the Unsaturation Index of plasma, RBC, and tissue fatty acids was unchanged, presumably because the increase of n-3 PUFA was counteracted by a decrease of n-6 PUFA. A slight but signifi-

TABLE 6
Phospholipid Fatty Acid Composition (% of total) of Lung Microsomal Membranes Isolated from Rats Treated with OA, EPA, and DHA (for 1 and 4 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Saturated	49.98 ± 2.23	47.60 ± 2.34	49.46 ± 1.67	48.82 ± 1.79	48.33 ± 2.30	46.28 ± 1.77
Monounsaturated	17.60 ± 0.93	17.15 ± 0.98	16.95 ± 2.54	18.26 ± 1.56	16.78 ± 1.08	16.10 ± 0.94
18:2n-6	10.34 ± 0.36	10.12 ± 0.31	10.35 ± 0.65	10.47 ± 0.08	11.24 ± 0.34	10.81 ± 0.27
18:3n-6	0.19 ± 0.02	0.23 ± 0.01	0.20 ± 0.05	0.21 ± 0.05	0.18 ± 0.02	0.20 ± 0.04
18:3n-3	0.29 ± 0.03	0.29 ± 0.02	0.26 ± 0.04	0.27 ± 0.01	0.29 ± 0.01	0.27 ± 0.05
20:3n-6	0.89 ± 0.08	0.93 ± 0.01	0.90 ± 0.18	0.98 ± 0.06	0.97 ± 0.06	0.90 ± 0.05
20:4n-6	12.16 ± 0.62	13.18 ± 1.16	11.63 ± 2.26	13.13 ± 0.82	11.04 ± 0.86	12.30 ± 0.87
20:5n-3	0.42 ± 0.09	0.53 ± 0.21	1.08 ± 0.21[a]	0.67 ± 0.13	0.59 ± 0.08	0.60 ± 0.16
22:4n-6	2.77 ± 0.34	2.98 ± 0.36	2.13 ± 0.20[a]	2.60 ± 0.33	2.02 ± 0.27[a]	2.34 ± 0.22
22:5n-3	1.57 ± 0.23	1.08 ± 0.20	3.03 ± 0.22[a,b]	2.49 ± 0.41[a,b]	1.24 ± 0.09	1.48 ± 0.08
22:6n-3	1.77 ± 0.16	1.87 ± 0.01	1.61 ± 0.27[b]	2.12 ± 0.25[b]	4.32 ± 0.80[a]	3.46 ± 0.03[a]
Total n-6 PUFA	26.33 ± 0.69	26.60 ± 2.12	25.69 ± 1.19	27.68 ± 1.96	26.12 ± 0.70	26.73 ± 1.53
Total n-3 PUFA	3.96 ± 0.34	3.58 ± 0.52	5.99 ± 0.50[a]	5.54 ± 0.41[a]	6.46 ± 0.94[a]	5.76 ± 0.19[a]
n-6/n-3 PUFA ratio	6.82 ± 0.52	6.65 ± 1.57	4.90 ± 0.68[a]	5.11 ± 0.63	4.50 ± 0.78a	4.87 ± 0.26
Unsaturation Index	121.99 ± 7.03	127.42 ± 10.75	126.19 ± 3.84	135.83 ± 6.98	132.85 ± 3.75	132.75 ± 3.56

[a] $P < 0.05$ vs. OA group, same week.
[b] $P < 0.05$ for difference between EPA and DHA group, same week.
[c] $P < 0.05$ vs. first week. For abbreviations, see Table 1. All values are means ± SE of five determinations.

TABLE 7
α-Tocopherol Content of Plasma, Red Blood Cell (RBC) Membranes, and Tissue Microsomal Membranes Isolated from Rats Treated with OA, EPA, and DHA (for 1 and 4 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Plasma (μM)	20.4 ± 2.0	19.3 ± 1.5	20.2 ± 1.3	20.9 ± 1.9	21.7 ± 1.1	22.9 ± 2.6
RBC membranes (ng/mg prot)	574.3 ± 44.7	544.0 ± 37.6	511.2 ± 41.1	443.9 ± 25.5[a]	757.0 ± 75.9[a,b]	461.5 ± 31.7[a,c]
Liver (ng/mg prot)	355.6 ± 31.1	284.0 ± 61.9	311.8 ± 20.7	299.4 ± 23.0	357.1 ± 32.0	287.7 ± 8.1
Kidney (ng/mg prot)	279.9 ± 16.0	282.9 ± 27.2	215.1 ± 15.4	237.2 ± 7.0	270.1 ± 29.3	227.4 ± 38.6
Heart (ng/mg prot)	555.6 ± 37.5	636.5 ± 64.5	509.9 ± 60.7	547.7 ± 79.7	425.1 ± 44.6[a]	632.0 ± 58.9
Lung (ng/mg prot)	1418.7 ± 158.6	1216.1 ± 150.9	1313.0 ± 102.9	1266.2 ± 102.2	1374.4 ± 137.8	1280.1 ± 92.2

[a] $P < 0.05$ vs. OA group, same week.
[b] $P < 0.05$ for difference between EPA and DHA group, same week.
[c] $P < 0.05$ vs. first week, same group. For abbreviations see Table 1. All values are means ± SE of five determinations.

cant increase in Unsaturation Index was observed only in heart membranes after 1 wk of DHA treatment (Table 5).

To evaluate whether the changes in fatty acid composition might account for increased susceptibility to lipid peroxidation, we exposed RBC isolated from animals treated with OA, EPA, and DHA for 4 wk to the prooxidant action of *t*-BOOH (Fig. 1A). RBC were incubated in the presence of 1 mM *t*-BOOH at 37°C for 90 min under oxygen. Lipid peroxidation was measured as malondialdehyde release. Neither EPA nor DHA treatments modified malondialdehyde release in RBC with respect to the control group. Under the same experimental conditions, we compared the time course of *t*-BOOH-induced hemolysis, measured as K^+ leakage, in RBC from OA, EPA, and DHA groups (Fig. 1B). *t*-BOOH-induced hemolysis did not differ in RBC isolated from the three groups.

In view of reports that fatty acid supplementation may influence vitamin E status (29), we analyzed the content of this antioxidant in plasma, RBC, and tissues after dietary treatment with OA, EPA, or DHA for 1 and 4 wk. The results are shown in Table 7. The three groups of animals did not differ in plasma, RBC, and tissue α-tocopherol content prior to dietary treatments. The highest level of α-tocopherol was found in lung, followed by heart, liver, RBC, and kidney. EPA did not induce any modification in α-tocopherol level after 1 wk, and a slight but significant decrease in RBC content was observed after 4 wk. DHA increased α-tocopherol content in RBC membranes after 1 wk and decreased it after 4 wk. Moreover, DHA treatment decreased α-tocopherol content in heart membranes after 1 wk and returned it to control values after 4 wk. This suggests a close relation between α-tocopherol content and Unsaturation Index modifications.

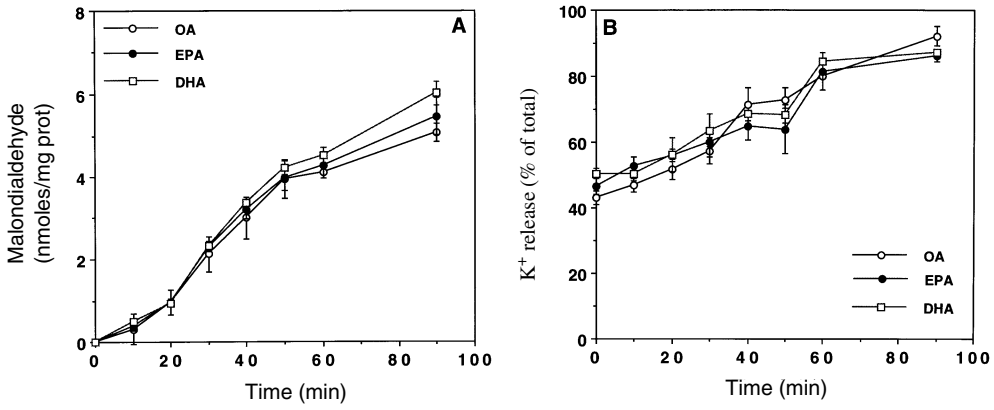

FIG.1. Lipid peroxidation and K^+ release induced by *tert*-butylhydroperoxide (*t*-BOOH) in red blood cells (RBC) isolated from rats treated with oleic acid (OA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) for 4 wk. RBC were incubated at 37°C for 90 min under oxygen in the presence of 0.5 mM *t*-BOOH. Lipid peroxidation (A) was measured as malondialdehyde formation. Intracellular K^+ release (B) was expressed as percentage of total efflux evoked by lysis of RBC in distilled water. Data are means ± SEM of five experiments.

DISCUSSION

The results show that the individual administration of low doses of EPA or DHA to rats does not change total lipid content but significantly modifies fatty acid composition of membrane phospholipids within 1 wk of treatment. The doses of n-3 PUFA in the present study were 2–10 times lower than those used by other authors (30–33) so as to approximate the dietary dose of the mixture of n-3 PUFA consumed in human studies (1,2,16,17). Beneficial effects of equivalent doses in human subjects at high risk for colon cancer (1,2) were not associated with any side effects (16,17). Moreover, the ratio n-3/n-6 PUFA used in the present study (1,2) was in the range (0.5–2) considered optimal for the expression of n-3 PUFA beneficial effects (34–36).

The changes in phospholipid composition in the rat membranes paralleled those observed in plasma and were directly related to the specific supplementation regimen. EPA was incorporated into the membranes, both directly and indirectly, as 22:5n-3 (DPA), its elongation product. This was reminiscent of the DPA accumulation in various tissues that occurs after fish oil administration (37,38). In agreement with our results, it has been reported that administration of purified EPA led to an increased level of DPA (31,32,39,40). This suggests that DPA represents a temporary storage site for surplus EPA, as postulated by Ackman (41).

Conversely, our data show that EPA supplementation did not lead to DHA (22:6n-3) increases in the tissues. This may be explained by an inhibitory effect of EPA on Δ6-desaturase (31,42) that has been postulated to be involved in the synthesis of 22:6n-3 from 22:5n-3 (38).

DHA treatment induced an increase in EPA (20:5n-3) in plasma, RBC, liver, and kidney. This effect was not observed in heart and lung, where DHA is presumably acylated to phospholipids rather than being retroconverted to 20:5n-3. Our data suggest a lowered capacity for retroconversion from 22:6n-3 to 20:5n-3 in tissues. This is in agreement with Gronn *et al.* (32), who reported a limited ability for retroconversion in heart. We reported earlier that retroconversion from 22:6n-3 to 20:5n-3 occurred in colon mucosa and transplanted hepatocarcinoma from rats (3,4).

Our results show increased levels of 20:5n-3 in plasma and in some membranes (lung, heart, and RBC) during EPA treatment. However, this increase was higher after 1 wk than after 4 wk. On the other hand, the increase of DHA observed in plasma and membranes was similar after 1 or 4 wk. Kinetics of incorporation of EPA and DHA in the various tissues probably reflects differences in metabolism and roles of EPA and DHA in biological membranes of specialized cells. For example it is known that DHA is generally present in mammary tissues at higher levels than EPA (43,44), which is consistent with our results. This observation relates to suggestions that DHA has a role in structural stability, whereas EPA is preferentially utilized for energy purposes, e.g., β-oxidation or eicosanoid synthesis (31,45,46).

Many studies with fish oils or purified EPA or DHA show that, as tissues become enriched in n-3 PUFA, they decrease their content in 20:4n-6 (arachidonic acid) and 18:2n-6 (linoleic acid) (31,38,40,47–49). However, in our experimental conditions, EPA or DHA at low concentrations decreased the content of 20:4n-6 in plasma and different tissues (liver, kidney, and heart) while increasing the level of 18:2n-6 in liver and kidney. This is best explained by the low dose used in our study, because with increasing doses of dietary n-3 PUFA, a large decrease of 18:2n-6 content occurs only with the highest doses (50).

The decrease of 20:4n-6 may be explained either by the known inhibition exerted by n-3 PUFA on the formation of 20:4n-6 from 18:2n-6 at the Δ6-desaturation step (51) or by the competition of n-3 PUFA with 20:4n-6 for acylation on the *sn*-2 position of membrane phospholipids (32). The first mechanism is likely to occur in liver, where Δ6-desaturation is a highly active process (48) and 18:2-n-6 levels are increased (Table 3). On the other hand, in heart, where Δ6-desaturation is limited (51) and 18:2n-6 levels do not change, the second mechanism is more plausible.

Although we found changes in plasma, RBC, and tissue membrane fatty acids as a consequence of EPA or DHA treatment, no significant modifications of Unsaturation Index were observed (Table 5). Only heart membranes showed a slight increase in the Unsaturation Index after 1 wk of treatment with DHA.

Our data show that dietary administration with low doses of n-3 PUFA can induce modifications of composition of phospholipid fatty acids without substantially affecting the degree of membrane unsaturation. This observation is consistent with what we found for α-tocopherol content.

α-Tocopherol content was not changed by EPA or DHA treatments. The only exception was heart membranes, where the treatment with DHA for 1 wk decreased the content of α-tocopherol while increasing the Unsaturation Index, suggesting that these two events are closely related. It is generally reported that dietary administration of n-3 PUFA increases lipid susceptibility for peroxidation, which is observed in membranes as an increase in Unsaturation Index and the consequent decrease of α-tocopherol content (52,53). The lack of change in this relationship found in our study suggests that dietary administration of low doses of n-3 PUFA does not modify susceptibility to oxidative stress.

Treatment with EPA and DHA did not modify the Unsaturation Index of RBC membranes. In contrast, α-tocopherol increased after 1 wk of treatment with DHA and decreased after 4 wk of treatment with both EPA and DHA. This confirms our previous data (16) and that of Berlin *et al.* (54) in human subjects. These studies found that short-term treatment with n-3 PUFA increased RBC membrane content of α-tocopherol and long-term treatment decreased it.

The modification of α-tocopherol in RBC could result from the peculiarity of these cells to freely exchange lipids between plasma and the cell membrane or from differential absorption of α-tocopherol induced by n-3 PUFA treatments.

In the present study, the treatments with EPA and DHA, at

the dose used, did not modify lipid peroxidation and hemolysis susceptibility of RBC exposed to an exogenous source of free radicals. Others reported that both parameters changed owing to oxidative stress induced by supplementation with n-3 PUFA (14,52). Our findings suggest that the overall susceptibility to lipid peroxidation of RBC is not increased and that the dose of n-3 PUFA used is an important variable in the induction of detrimental effects.

We conclude that EPA and DHA, administered at low doses and at an appropriate ratio to n-6 PUFA, modify membrane fatty acids in rat tissues without increasing the susceptibility to oxidative stress. This suggests that n-3 PUFA at low doses are appropriate for use in prevention and/or treatment of cardiovascular diseases and cancer, because detrimental secondary effects on cell membranes are unlikely.

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Molecular Speciation of Fish Sperm Phospholipids: Large Amounts of Dipolyunsaturated Phosphatidylserine

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ABSTRACT: The molecular species compositions of the main diacyl phosphoglyceride classes and ether-linked subclasses from sperm of three species of fish, sea bass *Dicentrarchus labrax,* Atlantic salmon *Salmo salar* and Chinook salmon *Onchorhynchus tsawytscha,* were determined. The phospholipids from sperm were highly unsaturated, dipolyunsaturated fatty acid (diPUFA) molecular species comprised 64.6 to 71.8% of phosphatidylserine (PS), 10.1 to 17.4% of phosphatidylethanolamine (PE), and 3.3 to 10.1% of phosphatidylcholine (PC). In sea bass sperm, di22:6n-3 phospholipid was the predominant diPUFA molecular species, but in both salmon species 22:5n-3/22:6n-3 was also a major constituent of PS. Phospholipids containing 22:6n-3 dominated in sea bass sperm with 16:0/22:6n-3 as a major component of PC and PE, and 18:0/22:6n-3 of PE and PS in addition to di22:6n-3 in the latter two classes. In contrast, both salmon species contained much more 20:5n-3 and less 22:6n-3 so that saturated/20:5n-3 and monounsaturated/20:5n-3 molecular species were more abundant than the corresponding molecules containing 22:6n-3. Ether-linked lipids comprised 11.3–36.3% of choline and ethanolamine phosphoglycerides in each fish species. Molecular species containing 22:6n-3 were the major components of 1-*O*-alkyl-2-acyl-glycerophosphocholine, especially 16:0a/22:6n-3 in sea bass and 18:1a/22:6n-3 in the two salmon species, while in 1-*O*-alk-1′-enyl-2-acyl-glycerophosphoethanolamine, 16:0a/22:6n-3 was the major component in both salmon and 18:0a/22:6n-3 in sea bass with 18:1a/22:6n-3 abundant in all three species. In Atlantic salmon 1-*O*-alkyl-2-acyl-glycerophosphoethanolamine comprised 24.6% of ethanolamine glycerophospholipids which were predominantly 16:0a/22:6n-3 and 18:1a/22:6n-3. Phosphatidylinositol from sperm was dominated by stearoyl/C_{20} PUFA molecular species, in sea bass overwhelmingly 18:0/20:4n-6, while in both salmon species 18:0/20:4n-6 and 18:0/20:5n-3 were equally abundant. *Lipids 32,* 1085–1091 (1997).

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Abbreviations: CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids; GPC, glycero-3-phosphocholine; GPE, glycero-3-phosphoethanolamine; HPTLC, high-performance thin-layer chromatography; MUFA, monounsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid. Molecular species are abbreviated as follows: e.g., 16:0/22:6 PC is 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine.

Phospholipids from sperm are rich in polyunsaturated fatty acids (PUFA) and, in most mammals, testes and sperm, together with brain and retina, are the main depositories of docosahexaenoic acid (22:6n-3) (1). PUFA accounted for 65–70% of fatty acids in total phospholipid from bull, boar and ram sperm, and about 40% in rabbit and human sperm (2). However, there is considerable variation between species in which PUFA are predominant. In rabbit, docosapentaenoic acid (22:5, isomer not specified) was the main PUFA with no 22:6n-3, and 22:5 was also abundant in boar sperm (2). In sperm from stallion and rat, 22:5 was also the predominant C_{22} PUFA (3,4), while rooster sperm was much more saturated (3). Ether-linked phosphoglycerides constitute a major part of choline glycerophospholipids (CGP) and ethanolamine glycerophospholipids (EGP) in sperm from mammals (5–7) and fish (8).

Phospholipids from fish tissues, and especially from marine fish, are generally more unsaturated than those from terrestrial mammals, particularly with respect to 20:5n-3 and 22:6n-3 (9,10). However, in a survey of the fatty acid composition of sperm from several species of fish from the Black Sea and freshwater, PUFA levels ranged from 26–55% in phosphatidylcholine (PC) and 26–66% in phosphatidylethanolamine (PE), similar to the levels found in mammals (8). Linoleic acid (18:2n-6) was found to be a major PUFA in fish from the Black Sea (8) which is unusual for marine fish. In farmed rainbow trout, total PUFA made up 33% of fatty acids in PC, 48% in PE and 49% in phosphatidylserine (PS) (11), and although 22:6n-3 was the predominant PUFA, levels of 18:2n-6 and 20:5n-3 were higher than expected for wild fish, reflecting dietary input. In sperm from wild sea bass, PUFA accounted for 35% of PC, 60% of PE and 85% of PS, predominantly 22:6n-3 (12). Farmed broodstock had similar levels of total PUFA and 22:6n-3, but ratios of arachidonic acid to eicosapentaenoic acid were substantially altered, reflecting a dietary input of fish oil rich in 20:5n-3 (12).

The very high amounts of PUFA, and especially 22:6n-3, in sperm suggest that amounts of diPUFA molecular species must be substantial in PE and PS. These unusual phospholipids, which are abundant in retina and to a lesser extent brain (13–16), also may be essential for sperm fertility and

confer protection from cold shock during cryopreservation. In order to gain a better understanding of the membrane structure of sperm, we determined the detailed molecular species composition of sperm phospholipid classes from wild sea bass (*Dicentrarchus labrax*), wild Atlantic salmon (*Salmo salar*), and wild Chinook salmon (*Oncorhynchus tsawytscha*).

MATERIALS AND METHODS

Experimental animals. Milt was obtained from freshly caught wild fish. Sea bass (*Dicentrarchus labrax*) were caught off Castellon, Spain, in February and March in a sea temperature of 15–17°C; Atlantic salmon (*Salmo salar*) were caught in the river Dee in Scotland in November and December in a water temperature of 5–7°C; and Chinook salmon (*Onchorynchus tsawytscha*) were caught off Neath Bay, Washington, USA, in a water temperature of 10°C. Samples were either frozen in liquid nitrogen or immediately mixed with chloroform/methanol (2:1, vol/vol) containing 0.01% (wt/vol) butylated hydroxytoluene, sealed under nitrogen, and stored at −20°C. Milt from three sea bass, three Atlantic salmon, and two Chinook salmon was used in the following experiments.

Chemicals and solvents. Phospholipase C from *Bacillus cereus* was purchased from Boehringer Corporation (London) Ltd. (Lewes, East Sussex, United Kingdom). Oxalyl chloride and anthracene-9-carboxylic acid were supplied by Aldrich Chemical Co. (Gillingham, Dorset, United Kingdom). All other chemicals and biochemicals were purchased from Sigma (Poole, Dorset, United Kingdom), and solvents of high-performance liquid chromatography grade were obtained from Labscan [B.S. & S.(Scotland) Ltd., Edinburgh, United Kingdom)].

Lipid extraction. Total lipid extracts were prepared as described previously (12). For molecular species analysis, equal amounts of total lipid from each fish were pooled for sea bass and Atlantic salmon. The milt from Chinook salmon was already pooled.

Determination of subclasses of CGP and EGP. CGP and EGP (1 mg) were digested with phospholipase C (80 units) in 1 mL 0.1 M borate buffer pH 7.5 and 1 mL diethyl ether for 1½ h under nitrogen at room temperature. Diradylglycerols were extracted and subclasses purified by high-performance thin-layer chromatography (HPTLC) in hexane/diethyl ether (1:1, vol/vol). The plate was sprayed with 0.1% (wt/vol) 2,7-dichlorofluorescein in 97% (vol/vol) methanol containing 0.01% (wt/vol) butylated hydroxytoluene and the bands were visualized under ultraviolet light. The bands were scraped from the thin-layer chromatography plate, 21:0 free fatty acid standard was added, and the mixture methylated for 16 h at 50°C under nitrogen with 2 mL 1% (vol/vol) H_2SO_4 in methanol and 1 mL toluene. Fatty acid methyl esters were extracted and purified as previously described (17) and chromatographed on a Packard 436 gas–liquid chromatograph fitted with a BP20 fused-silica capillary column (50 m × 0.32 mm, SGE) using hydrogen as carrier gas.

Preparation of anthroyl derivatives. Phospholipid classes (CGP, EGP, PS, and PI) were prepared by HPTLC in methyl acetate/chloroform/propan-2-ol/methanol/0.25% aq. KCl 25:25:25:10:9 (by vol). Purified phospholipids (200 µg) were digested with phospholipase C and 9-anthroyl derivatives prepared as described previously (18). For EGP, *N,N*-diisopropylethylamine was added as a proton scavenger to protect the acid-labile alkenyl linkage during the derivatization reaction (19). The 9-anthroyl diradyl derivatives were extracted, purified, and the diacyl, alkylacyl, and alkenylacyl subclasses separated by HPLTC in hexane/toluene/diethyl ether 4.5:4.0:0.3 (by vol).

Molecular species were separated by high-performance liquid chromatography using an Ultrasphere C_{18} column (25 × 0.46 cm, 5 µm particle size) (Beckman Instruments U.K. Ltd., High Wycombe, United Kingdom) and isocratic elution with acetonitrile/propan-2-ol (7:3, vol/vol) at a flow rate of 1.0 mL/min. Methanol/propan-2-ol (4:1, vol/vol) at a flow rate of 1.0 mL/min was employed to separate unresolved peaks. Peaks were detected using a Waters (Watford, United Kingdom) 470 scanning fluorescence detector (excitation 360 nm, emission 460 nm) and quantified with a Shimadzu CR3A recording integrator (Tokyo, Japan). Peaks were identified with reference to standard lipids and samples of known composition (13–15,20).

RESULTS

Ether-linked subclasses were minor components of CGP and EGP from fish sperm (Table 1). 1-*O*-Alk-1′-enyl-2-acyl-GPE comprised 11.3–23.5% of EGP in the three fish species, sea bass sperm containing the lowest proportion. Almost a quarter of EGP from Atlantic salmon sperm was the 1-*O*-alkyl-2-acyl subclass. This was absent in sperm from sea bass. In CGP, 15.5 and 19.2% of 1-*O*-alkyl-2-acyl-glycero-3-phosphocholine (GPC) was present in sea bass and Atlantic salmon, respectively (Table 1).

Molecular species containing 22:6n-3 dominated in PC, PE, and PS from sperm of sea bass (Table 2), with 22:6n-3/22:6n-3 a major component of PE (10.9%) and PS (50.2%), 16:0/22:6n-3 of PC (23.1%) and PE (31.2%), and 18:0/22:6n-3 of PE (13.9%) and PS (14.4%). In PE, 18:1/22:6n-3 was also prominent (8.5%), as was 22:5n-3/22:6n-3 in PS (9.0%). DiPUFA molecular species

TABLE 1
The Subclass Composition of CGP and EGP from Fish Sperm (wt%)[a]

	Subclass (%)		
	Diacyl	Alkylacyl	Alkenylacyl
Sea bass CGP	84.5	15.5	0
Sea bass EGP	88.7	0	11.3
Chinook salmon CGP	n.d.	n.d.	n.d.
Chinook salmon EGP	66.5	0	23.5
Atlantic salmon CGP	80.8	19.2	0
Atlantic salmon EGP	63.7	24.6	11.7

[a]The data were from a pooled sample from five, two, and three fish from sea bass, Chinook salmon, and Atlantic salmon, respectively. n.d. = not determined. CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids.

TABLE 2
Molecular Species Composition of the Diacyl Phosphoglycerides from Sperm of Sea Bass (mol%)[a]

Molecular species	PC	PE	PS	PI
20:5/22:6	0.8 ± 0.1	2.1 ± 0.3	3.6 ± 0.4	—
22:6/22:6	1.7 ± 0.1	10.9 ± 0.1	50.2 ± 0.6	—
22:5/22:6	0.2 ± 0.1	1.4 ± 0.0	9.0 ± 0.1	—
20:4/22:6	0.4 ± 0.1	1.9 ± 0.1	3.0 ± 0.1	—
22:5/22:5	0.3 ± 0.0	1.1 ± 0.1	6.0 ± 0.0	—
Total diPUFA	3.3	17.4	71.8	—
16:0/20:5	7.9 ± 1.8	4.7 ± 0.2	1.8 ± 0.1	0.6 ± 0.0
16:0/22:6	23.1 ± 1.9	31.2 ± 0.1	0.6 ± 0.1	0.3 ± 0.0
16:0/22:5	3.1 ± 0.0	4.8 ± 0.1	—	—
16:0/20:4	4.5 ± 0.1	2.7 ± 0.0	0.5 ± 0.0	3.1 ± 0.1
18:0/20:5	2.9 ± 0.0	4.0 ± 0.4	0.6 ± 0.0	6.1 ± 0.1
18:0/22:6	4.2 ± 0.2	13.9 ± 0.1	14.4 ± 0.9	2.0 ± 0.0
18:0/22:5	1.5 ± 0.0	0.5 ± 0.1	1.1 ± 0.1	0.5 ± 0.1
18:0/20:4	1.3 ± 0.0	2.2 ± 0.2	3.6 ± 0.2	71.5 ± 0.0
Total SFA/PUFA	48.4	64.0	22.6	84.2
16:1/22:6	1.3 ± 0.0	2.1 ± 0.1	—	—
16:1/22:5	1.3 ± 0.0	—	—	—
16:1/20:4	0.6 ± 0.0	0.3 ± 0.0	—	—
18:1/20:5	1.6 ± 0.0	0.9 ± 0.1	1.9 ± 0.1	0.2 ± 0.2
18:1/22:6	3.9 ± 1.8	8.5 ± 1.2	1.6 ± 0.5	—
18:1/22:5	1.1 ± 0.0	1.7 ± 0.1	—	—
18:1/20:4	—	—	—	3.7 ± 0.6
20:1/22:6	—	1.9 ± 0.2	0.9 ± 0.2	—
Total MUFA/PUFA	9.8	15.4	4.4	4.9
16:1/16:1	—	1.0 ± 0.1	—	—
16:0/16:0	1.3 ± 0.0	—	—	—
16:0/18:1	22.8 ± 0.5	1.0 ± 0.1	—	2.6 ± 0.0
18:1/18:1	2.0 ± 0.1	0.4 ± 0.0	0.6 ± 0.1	0.4 ± 0.0
18:0/18:1	1.1 ± 0.0	—	—	0.4 ± 0.0
18:1/20:1	0.2 ± 0.0	0.3 ± 0.1	0.5 ± 0.0	—
Total others	27.4	2.7	1.1	3.4

[a]The data are mean ± range of two determinations on a pooled sample from five fish. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids. The following fatty acids were identified by gas–liquid chromatography: 14:0, 15:0, 16:0, 17:0, 18:0, 16:1n-9 and n-7, 18:1n-9 and n-7, 20:1n-9 and n-7, 16:2, 16:3, 18:2n-6, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-6, 22:5n-3, and 22:6n-3. Molecular species containing minor fatty acids could not be resolved from major species nor could molecular species containing different fatty acid isomers be separated, e.g., 16:0/22:5n-6 from 16:0/22:5n-3. The order of fatty acids does not imply any stereospecificity on the glycerol. A range of 0.0 is <0.05%. — = not detected.

comprised 17.4% of PE and 71.8% of PS (Table 2). Other 22:6n-3-containing molecular species, present at less than 4%, included 20:5n-3/22:6n-3, 20:4n-6/22:6n-3, and 20:1/22:6n-3 (Table 2). Saturated/PUFA species containing 20:5n-3 and 22:5n-3 also made significant contributions to PC and PE. In contrast, phosphatidylinositol (PI) was dominated by 18:0/20:4n-6 (71.5%), with 16:0/20:4n-6 and 18:1/20:4n-6 contributing 3.1 and 3.7%, respectively (Table 2). The second-most abundant molecular species in PI was 18:0/20:5n-3 (6.1%); species containing 22:5n-3 and 22:6n-3 were minor components of PI (Table 2). Over a quarter of the PC existed as molecular species with no PUFA, predominantly 16:0/18:1 (Table 2). Such molecular species were very minor components of the other three diacyl phospholipid classes.

The diacyl phospholipids from Atlantic salmon sperm contained much more 20:5n-3 and much less 22:6n-3 than sperm from sea bass (Table 3). In PC, 16:0/20:5n-3 (16.8%) was slightly more abundant than 16:0/22:6n-3 (14.9%), and 18:1/20:5n-3 and 18:0/20:5n-3 were both more abundant than the corresponding 22:6n-3 species (Table 3), in contrast to sea bass. The largest component of PC was 16:0/18:1 (36.7%). In PE, 16:0/20:5n-3 (21.2%) and 18:1/20:5n-3 (17.5%) were the largest components, closely followed by 16:0/22:6n-3 (15.2%) and 18:1/22:6n-3 (10.2%). The only other molecular species present at over 4% were 18:0/20:5n-3 and 16:0/20:4 n-6. The largest component of diPUFA PE was di22:6n-3, but 20:5n-3/22:6n-3, 20:5n-3/22:5n-3, and 22:5n-3/22:6n-3 were of similar magnitude (Table 3), in contrast to sea bass where di22:6n-3 dominated. DiPUFA species again domi-

TABLE 3
Molecular Species Composition of Diacyl Phosphoglycerides of Sperm from Atlantic Salmon (mol%)[a]

Molecular species	PC	PE	PS	PI
20:5/20:5	0.3 ± 0.0	0.5 ± 0.0	1.8 ± 0.0	—
20:5/22:6	1.6 ± 0.0	2.0 ± 0.0	4.2 ± 0.0	—
22:6/22:6	1.3 ± 0.1	3.3 ± 0.1	24.1 ± 0.2	—
20:5/22:5	0.6 ± 0.0	1.4 ± 0.0	2.6 ± 0.0	—
22:5/22:6	0.4 ± 0.0	2.9 ± 0.0	31.9 ± 0.3	—
22:5/22:5	—	—	—	—
Total DiPUFA	4.2	10.1	64.6	—
16:0/20:5	16.8 ± 0.0	21.2 ± 0.4	1.0 ± 0.0	8.1 ± 0.3
16:0/22:6	14.9 ± 0.1	15.2 ± 0.0	1.6 ± 0.0	2.8 ± 0.1
16:0/22:5	0.9 ± 0.3	1.3 ± 0.1	0.4 ± 0.2	—
16:0/20:4	1.7 ± 0.0	5.8 ± 0.0	0.8 ± 0.0	8.5 ± 0.0
18:0/20:5	3.1 ± 0.0	6.0 ± 0.0	10.2 ± 0.1	31.7 ± 0.7
18:0/22:6	1.3 ± 0.0	1.2 ± 0.1	9.4 ± 0.1	2.7 ± 0.5
18:0/22:5	1.0 ± 0.0	—	1.2 ± 0.1	0.5 ± 0.0
18:0/20:4	0.3 ± 0.0	0.8 ± 0.0	2.4 ± 0.1	32.0 ± 0.4
Total SFA/PUFA	38.7	51.5	27.0	86.3
16:1/20:5	1.2 ± 0.0	2.4 ± 0.0	0.8 ± 0.0	0.3 ± 0.0
16:1/22:6	1.6 ± 0.0	2.4 ± 0.0	1.4 ± 0.0	—
16:1/22:5	0.7 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
16:1/20:4	0.5 ± 0.0	0.3 ± 0.0	—	0.4 ± 0.1
18:1/20:5	2.7 ± 0.0	17.5 ± 0.0	1.0 ± 0.0	1.9 ± 0.0
18:1/22:6	0.3 ± 0.4	10.2 ± 0.3	1.4 ± 0.0	0.5 ± 0.2
18:1/22:5	0.4 ± 0.0	1.0 ± 0.0	0.2 ± 0.0	0.9 ± 0.1
18:1/20:4	0.9 ± 0.0	0.2 ± 0.1	0.4 ± 0.0	3.4 ± 0.1
Total MUFA/PUFA	8.3	34.2	5.5	7.7
16:0/16:0	1.4 ± 0.0	—	0.3 ± 0.0	0.5 ± 0.1
16:0/18:1	36.7 ± 0.1	0.4 ± 0.1	1.6 ± 0.1	0.2 ± 0.0
18:1/18:1	0.9 ± 0.0	—	0.2 ± 0.0	0.2 ± 0.0
18:0/18:1	1.2 ± 0.0	—	—	—
Total others	40.2	0.4	2.1	0.9

[a]Data are mean ± range of two determinations on a pooled sample from three fish. See Table 2 footnote for abbreviations and fatty acid identification information.

nated PS, although the total was lower than in sea bass, and 22:5n-3/22:6n-3 (31.9%) was the largest component followed by di22:6n-3 (24.1%), with 20:5n-3/20:5n-3, 20:5n-3/22:6n-3, and 20:5n-3/22:5n-3 each contributing 1.8–4.2% (Table 3). The only other species present at more than 3% were 18:0/20:5n-3 and 18:0/22:6n-3, both about 10% (Table 2). PI was again dominated by saturated fatty acid (SFA)/PUFA species, with 18:0/20:4n-6 (32.0%) and 18:0/20:5n-3 (31.7%) equally abundant; 16:0/20:4n-6 (8.5%) and 16:0/20:5n-3 (8.1%) were the only other molecular species present at greater than 4%. No diPUFA species were found in PI, and only 0.9% of species containing only SFA or MUFA was present (Table 3).

The molecular species composition of phospholipids from Chinook salmon sperm was generally similar to that of Atlantic salmon with 20:5n-3-containing molecular species being major components (Table 4). In the SFA/PUFA and monounsaturated fatty acids (MUFA)/PUFA categories of PC and PE, 16:0, 18:0 and 18:1 species containing 20:5n-3 were all more abundant than the corresponding species containing 22:6n-3 (Table 4). DiPUFA molecular species comprised a larger proportion of PC, PE, and PS in Chinook than in Atlantic salmon. Although 22:5n-3/22:6n-3 was again abundant in PS (23.8%), di22:6n-3 was the largest component (35.4%) (Table 4). In PI, 18:0/20:5n-3 and 18:0/20:4n-6 were again almost equally abundant (33.4 vs. 30.0%, respectively) but 18:0/22:6n-3 made up 15.3% of PI from Chinook salmon sperm (Table 4).

The differences noted in the molecular speciation of the diacyl phospholipids in the three fish species were also apparent in the ether-linked subclasses. 1-*O*-Alkyl-2-acyl-GPC from sea bass was dominated by 16:0a/22:6n-3 (40.3%), with 18:1a/20:5n-3 the only other molecular species over 10% (Table 5). Molecular species comprised only of SFA or MUFA totaled 3.8% of the sample. The three molecular species containing 20:4n-6 totaled 12.2% in sea bass compared with only 4.0% in Chinook salmon and 1.1% in Atlantic salmon (Table 5). In Chinook salmon sperm, 16:0a/22:6n-3, 18:1a/20:5n-3, 18:1a/22:6n-3, and 16:0a/18:1 1-*O*-alkyl-2-acyl-GPC each comprised 11.6–25.2% of the sample (Table 5). The same four molecular species also dominated the 1-*O*-alkyl-2-acyl-GPC subclass from Atlantic salmon sperm (Table 5). While 16:0a/22:6n-3 and 18:0a/22:6n-3 were less abundant in both salmon species than in sea bass 1-*O*-alkyl-2-acyl-GPC, the converse was the case for 18:1a/22:6n-3, this largely contributing to the increase in the 18:1a/PUFA category of molecular species in Chinook and Atlantic salmon compared to sea bass (Table 5). In 1-*O*-alkyl-2-acyl GPE from sperm of Atlantic salmon, 16:0a/22:6 + 18:1a/22:6 totaled over 59%, and the corresponding 20:5n-3-containing molecular species totaled almost 27% (Table 5). In contrast to 1-*O*-alkyl-2-acyl GPC, 16:0a/18:1 only comprised 0.8% of 1-*O*-alkyl-2-acyl GPE.

In 1-*O*-alk-1′-enyl-2-acyl-GPE, the three main molecular species in sea bass sperm were 18:0a/22:6n-3, 18:1a/22:6n-3 and 16:0a/22:6n-3, which together totaled 75.0% of the sample (Table 6). In the two salmon species 16:0a/22:6n-3 1-*O*-alkenyl-2-acyl-GPE was more prominent than in sea bass while 18:0a/22:6n-3 was much less prominent: *ca.* 2% compared with 33.4% in sea bass. Amounts of 18:1a/22:6n-3 were similar in all three species of fish (Table 6). In contrast 16:0a/20:5n-3 and 18:1a/20:5n-3 were much more abundant in the salmons while 18:0a/20:5n-3 comprised only 1.0% of Chinook salmon alkenylacyl-GPE and was not detected in Atlantic salmon (Table 6). The 18:0a alkenyl chains were much more abundant in sea bass than in either salmon, as were 18:1a/20:4n-6 and 18:1a/22:5n-3. Molecular species containing just SFA or MUFA were very minor constituents of alkenylacyl-GPE in sperm from all three fish (Table 6).

TABLE 4
The Molecular Species Composition of the Diacyl Phosphoglycerides from Sperm of Chinook Salmon (mol%)

Molecular species	PC	PE	PS	PI
20:5/20:5	1.2 ± 0.0	0.5 ± 0.0	0.8 ± 0.0	—
20:5/22:6	3.6 ± 0.0	3.1 ± 0.0	5.7 ± 0.0	—
22:6/22:6	1.3 ± 0.0	4.7 ± 0.0	35.4 ± 0.2	—
20:5/22:5	1.0 ± 0.0	1.7 ± 0.1	0.4 ± 0.0	—
22:5/22:6	1.9 ± 0.0	2.1 ± 0.0	23.8 ± 0.1	—
22:5/22:5	1.0 ± 0.0	3.4 ± 0.0	0.6 ± 0.0	—
Total DiPUFA	10.1	15.6	66.7	—
16:0/20:5	27.2 ± 0.0	17.8 ± 0.2	1.5 ± 0.1	5.5 ± 0.2
16:0/22:6	14.2 ± 0.1	7.5 ± 0.0	0.8 ± 0.0	3.1 ± 0.0
16:0/22:5	2.2 ± 0.1	3.9 ± 0.0	1.0 ± 0.0	1.1 ± 0.2
16:0/20:4	1.8 ± 0.0	4.3 ± 0.0	0.7 ± 0.1	4.1 ± 0.0
18:0/20:5	1.6 ± 0.0	4.9 ± 0.0	9.6 ± 0.0	33.4 ± 0.4
18:0/22:6	0.3 ± 0.0	0.9 ± 0.1	8.6 ± 0.1	15.3 ± 0.2
18:0/22:5	2.9 ± 0.0	—	1.0 ± 0.1	0.7 ± 0.1
18:0/20:4	0.3 ± 0.1	0.6 ± 0.0	2.8 ± 0.1	30.0 ± 0.3
Total SFA/PUFA	50.5	39.9	25.9	93.1
16:1/20:5	0.5 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	—
16:1/22:6	1.8 ± 0.0	2.8 ± 0.0	0.8 ± 0.0	—
16:1/22:5	—	0.3 ± 0.0	—	—
16:1/20:4	—	0.4 ± 0.0	—	—
18:1/20:5	4.0 ± 0.0	23.9 ± 0.0	2.3 ± 0.0	2.1 ± 0.2
18:1/22:6	1.2 ± 0.1	12.9 ± 2.1	3.5 ± 0.0	0.2 ± 0.1
18:1/22:5	0.3 ± 0.0	0.9 ± 0.0	0.4 ± 0.0	1.1 ± 0.0
18:1/20:4	—	—	—	2.0 ± 0.2
20:1/22:6	—	—	0.5 ± 0.3	—
Total MUFA/PUFA	7.7	41.7	7.8	2.0
16:0/16:0	4.2 ± 0.0	—	—	0.5 ± 0.0
16:0/18:1	21.6 ± 0.1	—	0.2 ± 0.0	—
18:1/18:1	0.7 ± 0.1	—	—	—
18:0/16:0	0.2 ± 0.0	—	—	—
18:0/18:1	1.2 ± 0.0	—	—	—
Total others	27.8	—	0.2	0.5

[a]Mean ± range of two determinations on a pooled sample from two fish. See Table 2 footnote for abbreviations and fatty acid identification information.

DISCUSSION

In mammals, ether-linked subclasses are the major components of sperm phospholipids. The alkenylacyl subclass com-

TABLE 5
The Molecular Species Composition of 1-*O*-Alkyl-2-acyl-glycerophospholipid from Fish Sperm (mol%)[a]

	Sea bass	Chinook salmon	Atlantic salmon	
Molecular species	GPC	GPC	GPC	GPE
16:0a/20:5	6.5 ± 0.1	8.1 ± 0.1	9.0 ± 0.5	10.9 ± 0.5
16:0a/22:6	40.3 ± 0.0	25.2 ± 0.1	23.9 ± 0.0	30.4 ± 0.2
16:0a/22:5	—	—[b]	1.1 ± 0.1	0.1 ± 0.1
16:0a/20:4	6.4 ± 0.0	1.5 ± 0.0	0.8 ± 0.1	1.0 ± 0.2
16:0a/18:2	2.7 ± 0.1	3.4 ± 0.0	—	—
18:0a/20:5	3.9 ± 0.0	0.8 ± 0.0	5.8 ± 0.1	1.3 ± 0.4
18:0a/22:6	8.0 ± 0.1	1.4 ± 0.0	0.8 ± 0.1	1.2 ± 0.7
18:0a/22:5	0.2 ± 0.0	0.6 ± 0.0	—	—
18:0a/20:4	0.6 ± 0.1	—	—	—
Total	68.6	41.0	41.4	44.9
18:1a/20:5	10.2 ± 0.1	11.6 ± 0.1	16.4 ± 0.1	15.9 ± 0.0
18:1a/22:6	4.9 ± 0.1	23.6 ± 0.6	20.7 ± 0.1	28.8 ± 0.5
18:1a/22:5	3.9 ± 0.1	1.8 ± 0.0	1.0 ± 0.1	4.3 ± 0.3
18:1a/20:4	5.2 ± 0.0	2.3 ± 0.0[b]	0.3 ± 0.1	1.2 ± 0.2
18:1a/18:2	0.9 ± 0.1	—	—	—
Total	25.1	39.5	38.4	50.2
16:0a/16:0	0.2 ± 0.0	0.8 ± 0.0	0.5 ± 0.1	—
16:0a/18:1	2.7 ± 0.0	17.1 ± 0.1	14.1 ± 0.0	0.8 ± 0.0
18:1a/18:1	0.9 ± 0.0	0.4 ± 0.1	0.8 ± 0.0	0.9 ± 0.2
Total	3.8	18.3	15.4	1.7

[a]Mean ± range of two determinations on pooled samples from five, two, and three fish, respectively.
[b]16:0a/22:5 and 18:1a/20:4 could not be resolved in this sample. See Table 2 footnote for fatty acid identification information. GPC, glycero-3-phosphocholine; GPE, glycero-3-phosphoethanolamine.

TABLE 6
The Molecular Species Composition of 1-*O*-Alk-1′-enyl-2-acyl GPE from Fish Sperm (mol%)[a]

Molecular species	Sea bass	Chinook salmon	Atlantic salmon
16:0a/20:5	0.4 ± 0.0	9.9 ± 0.2	11.4 ± 0.4
16:0a/22:6	14.5 ± 0.1	38.1 ± 0.2	40.5 ± 0.1
16:0a/22:5	—	3.0 ± 0.0	0.4 ± 0.0
16:0a/20:4	1.0 ± 0.1	0.9 ± 0.0	0.6 ± 0.1
17:0a/22:6	2.0 ± 0.1	—	—
18:0a/20:5	3.2 ± 0.3	1.0 ± 0.0	—
18:0a/22:6	33.4 ± 1.5	2.2 ± 0.1	1.8 ± 0.0
18:0a/22:5	2.0 ± 0.2	0.2 ± 0.1	—
18:0a/20:4	1.4 ± 0.0	0.2 ±0.1	1.0 ± 0.0
Total	58.1	55.5	55.7
18:1a/20:5	0.2 ± 0.0	9.8 ± 0.0	6.2 ± 0.2
18:1a/22:6	26.1 ± 0.9	24.6 ± 0.3	32.2 ± 3.6
18:1a/22:5	4.2 ± 0.4	2.2 ± 0.0	0.9 ± 0.1
18:1a/20:4	5.4 ± 0.2	1.3 ± 0.2	0.7 ± 0.2
Total	35.8	37.9	40.0
16:0a/16:0	0.2 ± 0.0	—	—
16:0a/18:1	0.3 ± 0.0	0.6 ± 0.1	—
18:1a/18:1	0.4 ± 0.0	0.4 ± 0.1	—
Total	0.9	1.0	—

[a]Mean ± range of two determinations on pooled samples from five, two, and three fish, respectively. See Table 2 footnote for fatty acid identification information. See Table 5 for abbreviation.

prised 56% of EGP and 70% of CGP in caudal sperm from goat (7), while slightly lower proportions were found in rat and porcine sperm (5,6). Alkylacyl moieties comprised *ca.* 20% of CGP in goat and *ca.* 50% in porcine sperm, and *ca.* 25% of EGP in goat and *ca.* 50% in porcine sperm (6,7). The amounts of alkenylacyl-GPE found in this study were lower than those reported by Drokin (8) in several fish species from the Black Sea (19.7–46.1%) and freshwater (17.2–46.4%). In mammals, the proportion of ether-linked CGP and EGP increases during maturation (5–7), and this could account for the difference. Changes in the phospholipid class composition during sperm maturation have not been investigated in fish, and it is possible that the proportion of ether-linked phospholipids found in this study would have been higher had sperm been collected during natural spawning rather than by stripping. We did not find alkenylacyl-GPC as found by Drokin (8) who did not determine the content of alkylacyl-GPC.

The phospholipids from all three sperm samples were very unsaturated, with almost all molecular species from PE, PS, and PI containing at least one polyunsaturated acyl chain. Only in PC were species containing only SFA and MUFA major components. PS contained by far the largest amount of diPUFA species, with over half the sample di22:6n-3 in sea bass, over a third in Chinook salmon, and a quarter in Atlantic salmon; 22:5n-3/22:6n-3 was also a major component of PS from both salmon species. The specificity for di22:6n-3 in the diPUFA category of PE was again much less marked in the salmon. PI was typically dominated by stearoyl/PUFA species, but there was a marked difference between the sea bass and the two salmon species, sea bass overwhelmingly containing 18:0/20:4n-6, while in the salmon 18:0/20:5n-3 was the largest component and 18:0/22:6n-3 also contributed significantly. Though arachidonic acid is a minor PUFA in many fish species, it is concentrated in PI (21). In retina of trout, and in retina, liver and muscle of cod, 18:0/20:4n-6 was the major molecular species, but in brain of both species 18:0/20:5n-3 was predominant (13,15). Salmon sperm is therefore rather unusual in having so much 18:0/20:5n-3 PI. Few other studies have detemined the molecular species composition of sperm phospholipids. In PC and PE from rat testes, only small amounts (<2%) of diPUFA species (20:4/20:4 and 18:2/20:4) were found, but these two molecular species showed the fastest metabolic turnover (4).

Sea bass have a more southern distribution than either Atlantic or Chinook salmon and are therefore found in warmer waters. It has been proposed that 18:1/22:6n-3 PE is a key molecular species in thermal adaptation of membranes in fish (22–24). It was therefore noteworthy that in this study the MUFA/PUFA category of PE molecular species showed one of the largest differences between sperm from sea bass and sperm from the two salmonids, being more than doubled in the salmonids with a concomitant drop in SFA/PUFA molecular species. However, because of the abundance of 20:5n-3 in the salmonids, most of this increase was due to 18:1/20:5n-3; 18:1/22:6n-3 PE was only slightly elevated in the salmonids compared to the sea bass. These differences

were not apparent in 1-*O*-alk-1′-enyl-2-acyl-GPE. In addition there was little difference in MUFA/PUFA PC in the three fish species.

The plasma membrane of mammalian sperm cells contains regions of nondiffusing lipid resulting from coexistent fluid and gel domains (25). Mammalian sperm encounters some variation in temperature during maturation, and lipid phase transitions may be important for stabilizing the membranes during epididymal storage and during fertilization. It has been suggested that temperature changes might play a role in the mammalian fertilization process by altering membrane phase behavior (26). Sperm from poikilotherms is not exposed to temperature change during fertilization, but in salmonids there will usually be a temperature change during migration of the fish from seawater to fresh water and thus during sperm maturation. It would be interesting to know whether the plasma membrane of sperm from fish has the same lipid organization as sperm from mammals.

The essential role of 22:6n-3 in biomembrane function has attracted considerable attention in recent years, but mainly has been concerned with the role of 22:6n-3 in neural tissue (e.g., 27–29). A deficiency of 22:6n-3 during early development when neural membranes are being formed caused visual and cognitive subperformance in rats, rhesus monkeys, and humans (30–32) and visual impairment in juvenile herring (18). Reconstitution experiments with rhodopsin and phospholipids containing 22:6n-3 have shown that it is a combination of PC, PE, and PS head groups and at least 50% docosahexaenoyl chains that are required to give full activity (see Ref. 33). Molecular modeling studies have suggested that 22:6n-3 has favored conformations (angle-iron or helix) (34,35) which give phospholipids containing this fatty acid unusual packing properties (36). Docosahexaenoic acid also was found to have the smallest volume change with temperature of any fatty acid (35). Sperm are relatively resistant to cold shock (37), and the special properties of 22:6n-3-containing phospholipids could be crucial during cryopreservation as well as being important in thermal adaptation processes in general. Phospholipids with small head groups and two PUFA chains (i.e., di22:6n-3 PE) or polyunsaturated anionic lipids (i.e., PS) give unstable lipid bilayers which revert to reverse hexagonal phases (33). The tendency of such lipids with negative interfacial curvature to form nonlamellar phases is hypothesized to lower the activation energy of the MI-MII transition in rhodopsin (33). Such lipids are also fusogenic (38) and could therefore be important in aiding or promoting fusion between the plasma membrane of eggs and sperm. In guinea pig sperm, anionic-lipid concentrations increased as the membrane became fusionally competent (39). It was found that 18:1/22:6 PE was more effective than other more saturated PE species at lowering the bilayer to hexagonal phase transition, thus enhancing the activity of protein kinase C (40). PE plasmalogens also destabilize the lamellar phase and promote the formation of hexagonal phase, this transition occurring at lower temperatures in 1-*O*-alkenyl-2-acyl GPE than in 1-*O*-alkyl-2-acyl or diacyl GPE (41 and references therein). Perhaps the lower but more constant temperature to which fish sperm are exposed necessitates a smaller proportion of ether-linked phospholipids in order to maintain the lamellar phase found in normal cell membranes.

In humans, sperm density, number of motile sperm, and sperm motility were all correlated with the amount of 22:6n-3 (42), strongly suggesting a role for 22:6n-3 in sperm motility and therefore in fertility. It was especially noteworthy in this study that, in the two salmonids, 22:5n-3 was able to replace 22:6n-3 to a considerable degree in both diPUFA PS and PE. Large amounts of 22:5 were found in sperm from rabbit, boar, rat, stallion and rooster, together with lower amounts of 22:6n-3 (2,3). Therefore, in sperm it appears that 22:5 can substitute functionally for 22:6n-3. This is not the case in neural tissue where neither 22:5n-3 nor 22:5n-6 is able to substitute for 22:6n-3 (18,28). In spite of changes in the relative contributions of 22:6n-3, 22:5n-3 and 20:5n-3 in the different phospholipid classes in the three fish species, considerable constancy in the various categories of molecular species existed within the phospholipid classes, suggesting specific functional requirements at this level of membrane organization. It would be interesting to know whether or not the same pattern of lipid molecular speciation is found in the sperm of homeotherms. As readily available single cells, sperm may be a good experimental system for unraveling the essential roles of C_{22} PUFA in membrane function.

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Lipids and Buoyancy in Southern Ocean Pteropods

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ABSTRACT: The lipids of *Clione limacina,* a Southern Ocean pteropod (order Gymnosomata), contain 28% diacylglyceryl ether (DAGE) (as percentage of total lipid) whereas the pteropod *Limacina helicina* (order Thecosomata) lacks DAGE. The alkyl glyceryl ether diols (1-*O*-alkyl glycerols, GE) of *Clione* DAGE are dominated by 16:0 (60%) and 15:0 (21%), in contrast with deep-sea shark liver DAGE, which is dominated by 18:1 GE. The fatty acid profiles of *Clione* and *Limacina* are similar (28–32% polyunsaturated, 26–34% monounsaturated) as are the sterols, which include 24-methylenecholesterol, transdehydrocholesterol, cholesterol, and desmosterol. This finding probably reflects the fact that *Limacina* is the major food source for *Clione. Spongiobranchaea australis,* another Southern Ocean pteropod (order Gymnosomata), has 0.9–1.7% DAGE, but has less lipid (3.3–4.8 mg/g lipid, wet weight) than *Clione* (50.8 mg/g lipid, wet weight). We propose a buoyancy role for DAGE in *Clione* since *Limacina* has bubbles for flotation which *Clione* lack; DAGE provides 23% more uplift than triacylglycerol at a concentration of 1.025 g/mL seawater.
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Marine zooplankton maintain buoyancy by diverse strategies including hypotonicity, increased surface area, swimming, gas bubbles, and storage of low-density materials such as lipid (0.86–0.93 g/mL). The most important storage lipids in marine zooplankton are wax esters (WE) and triacylglycerol (TAG) (1). WE may be concentrated in polar and deep ocean copepods, where they are utilized primarily as long-term energy reserves (2). TAG, however, are major short-term energy reserve molecules in most zooplankton. WE have a lower metabolic turnover rate than TAG, and thus may have a more important function in buoyancy than TAG (3). The density of WE (0.86 g/mL at 21°C) is less than TAG (0.93 g/mL at 21°C). Diacylglyceryl ether (DAGE), although not a major lipid in zooplankton, also has a lower metabolic turnover rate than TAG and a lower density (0.89 g/mL at 21°C). DAGE also generates 23% more upthrust than TAG (0.127 g upthrust/g lipid vs. 0.103 g upthrust/g lipid in seawater of 1.025 g/mL density) (4).

Lipid composition is also used to understand and identify food web interactions (5,6). The Southern Ocean has a complex food web including phytoplanktivorous herbivores fed upon by birds, fish, squid, seals, and baleen whales (7). Sterols (ST) and fatty acids are excellent biomarkers for food chain studies (8). The herbivorous Thecosome pteropod *Limacina helicina* is very common in the Southern Ocean, as in other oceans (9). It is fed upon by the carnivorous Gymnosome pteropod, *Clione limacina,* another abundant holoplanktonic Opisthobranch mollusk. *Spongiobranchaea australis* is another highly predaceous Gymnosome pteropod, which also feeds exclusively on a Thecosome pteropod (*Clio pyramidata*).

Limited lipid compositional data are available for these Southern Ocean pteropods. The purpose of this study is to report the use of DAGE as a possible buoyancy lipid in the pteropod *C. limacina,* and to clarify and further understand food web interactions by examining pteropod ST and fatty acids as key biochemical factors.

MATERIALS AND METHODS

Sample description. The pteropods *C. limacina* and *L. helicina* were collected by Isaacs-Kidd midwater trawl from the R/V *Yuzhmorgeologia* on January 23 and 22, l996, respectively. These samples were obtained as part of the Antarctic Marine Life Research Field Study conducted annually in the Elephant Island region of the Antarctic peninsula (10,11). *Clione limacina* was trawled from station A-04 (off Admiralty Bay, King George Island) at 62°14.4´ S and 57°58.5´ W. *Limacina helicina* was trawled from station AB-03 (within Admiralty Bay). In both cases the net was towed to 170 m depth for about 30 min. Pteropods were frozen at –10°C as soon as possible after sorting and counting on board ship. They were then transported frozen by air to the CSIRO Division of Marine Research where they were maintained at –60°C prior to analysis. *Spongiobranchaea australis* was collected by rectangular midwater trawl (to 195 m) from the R/V *Aurora Australis* on Expedition Broke on March 12, l996, from the Adélie coast (within Wilkes land) region of East Antarctica (63°26.27´ S and 138°59.21´ W). They were

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Abbreviations: DAGE, diacylglyceryl ether; FFA, free fatty acids; FID, flame-ionization detector; GC, gas chromatography; GE, 1-*O*-alkyl glycerols; MS, mass spectrometric; PL, phospholipid; PUFA, polyunsaturated fatty acids; ST, sterols; TAG, triacylglycerols; TLC, thin-layer chromatography; TMS, trimethylsilyl; WE, wax esters.

frozen in liquid nitrogen immediately and transported frozen to the CSIRO Hobart laboratory for analysis. Individuals of *C. limacina* were pooled for extraction owing to the limited sample size (8–9 individuals weighed 0.25 g wet weight). It was possible to pool 20–25 individuals of *L. helicina* for three extractions owing to a larger sample size. Duplicate analyses of two individuals of the considerably larger *S. australis* were performed.

Lipid extraction. Samples were quantitatively extracted using a modified Bligh and Dyer (12) one-phase methanol/chloroform/water extraction (2:1:0.8, by vol); the sample was extracted overnight and the phases were separated the following day by the addition of chloroform and water (final solvent ratio, 1:1:0.9, by vol, methanol/chloroform/water). The total solvent extract was concentrated (i.e., solvents removed *in vacuo*) using rotary evaporation at 30°C. Lipid class analyses were conducted immediately; for other purposes samples were stored for no more than 3 d in a known volume of chloroform.

Lipids. An aliquot of the total solvent extract was analyzed using an Iatroscan MK V TH10 thin-layer chromatography–flame-ionization detector (TLC–FID) analyzer (Tokyo, Japan) to determine the abundances of individual lipid classes (13). Samples were applied in duplicate or triplicate to silica gel SIII Chromarods (5-μm particle size) using 1 μL disposable micropipets. Chromarods were 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 (60:17:0.2, by vol), a mobile phase resolving nonpolar compounds such as WE, TAG, free fatty acids (FFA), and ST. A second nonpolar solvent system of hexane/diethyl ether (96:4, vol/vol) was also used to separate hydrocarbons from WE and TAG from diacylglycerol. After development, the chromarods were oven-dried and analyzed immediately to minimize adsorption of atmospheric contaminants. The FID was calibrated for each compound class (phosphatidylcholine, cholesterol, cholesteryl ester, oleic acid, squalene, triolein, and DAGE purified from shark liver oil; 0.1–10 μg range). A laboratory standard of WE was used for peak identification, and steryl ester was used for quantification of WE. Based on the TLC–FID analyses and subsequent analysis of component fatty acids and alcohols by gas chromatography (GC), steryl esters were either absent or only present as trace components. WE and steryl esters coelute in the systems used, but results for combined TLC–FID and GC assays indicated they were either absent or only present as trace components. Peaks were quantified on an IBM-compatible computer using DAPA software (Kalamunda, Western Australia). Iatroscan results are generally reproducible to ±10% (13).

*Fatty acids and 1-*O*-alkyl glycerols (GE).* An aliquot of the total solvent extract was treated with potassium hydroxide in methanol (5% wt/vol) and saponified under nitrogen for 3 h at 80°C. Nonsaponifiable neutral lipids (e.g., glyceryl ether diols and hydrocarbons) were then extracted into hexane/chloroform (4:1 vol/vol, 3 × 1.5 mL) and transferred to sample vials. Following acidification of the remaining aqueous layer using hydrochloric acid (pH = 2), fatty acids were extracted and methylated to produce their corresponding fatty acid methyl esters using methanol/hydrochloric acid/chloroform (10:1:1, by vol; 80°C, 2 h). Products were then extracted into hexane/chloroform (4:1 vol/vol, 3 × 1.5 mL) and stored at −20°C. The nonsaponifiable neutral lipid fractions were treated with *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (50 μL, 60°C, 1 h) to convert ST to their corresponding trimethylsilyl (TMS) ethers.

GC analyses of methyl esters of fatty acids were performed with a Hewlett-Packard 5890A GC (Avondale, PA) equipped with an HP-1 cross-linked methyl silicone fused silica capillary column (50 m × 0.32 mm i.d.), an FID, a split/splitless injector, and an HP 7673A auto sampler. Hydrogen was the carrier gas. Following addition of a methyl tricosanoate internal standard, samples were injected 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, then to 250°C at 2°C/min, and finally to 300°C at 5˚C/min. Peaks were quantified with DAPA Scientific Software. Individual components were identified using mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. GC results are subject to an error of ±5%.

GC–mass spectrometric (GC–MS) analyses were performed on a Fisons MD 800 GC–mass spectrometer (Manchester, United Kingdom) fitted with an on-column injector. The GC was fitted with a capillary column similar to that described above.

The Iatroscan (TLC–FID) analyzer provides a rapid means of detecting DAGE without any sample treatment and derivatization prior to analysis. To further confirm and elucidate the composition of the DAGE fraction, the process of saponification was used in this study to convert the DAGE to the corresponding GE. The GE are then extracted with other nonsaponifiable neutral lipid material and converted to di-*O*-TMS ether derivatives prior to analysis by GC. GC–MS analysis of the samples readily identified the GE components from their base peak at $m/z = 205$.

Determination of double-bond configuration in fatty acids. Dimethyl disulfide adducts of monounsaturated fatty acids were formed by treating the total fatty acid methyl esters with dimethyl disulfide (14,15). Adducts were then extracted using hexane/chloroform (4:1, vol/vol) and treated with *N,O*-bis-(trimethylsilyl)-trifluoroacetamide to form TMS derivatives prior to GC–MS analysis.

RESULTS

Lipid composition. Clione limacina (order Gymnosomata) has 27.8% DAGE, *S. australis* (order Gymnosomata) has 0.9–1.7% DAGE, and *L. helicina* (order Thecosomata) has only trace amounts of DAGE (Table 1). *Clione limacina* also has over 10 times as much lipid as the other two pteropods (50.8 mg/g, wet weight) (Table 1). The other major lipid classes in *C. limacina* include TAG (22.6%), FFA (23.9%),

TABLE 1
Composition of Lipids from Southern Ocean Pteropods

Sample	Percentage composition[a] (of total lipids)								Lipid (mg/g)[b]
	WE	TAG	FFA	ST	PL	UN	GE	Total	
Clione limacina	0.8	22.6	23.9	2.5	22.1	0.3	27.8	100.0	50.8
Limacina helicina	trace	—	54.9	57.7	39.4	—	trace	100.0	2.4
	0.4	—	59.1	6.3	34.2	—	trace	100.0	6.9
	0.2	—	57.1	6.7	36.0	—	trace	100.0	4.3
Spongiobranchaea	0.2	66.7	0.8	0.7	30.0	—	1.7	100.0	3.3
australis	0.2	39.5	0.4	0.9	58.1	—	0.9	100.0	4.8

[a]Abbreviations: WE, wax ester; TAG, triacylglycerol; FFA, free fatty acid; ST, sterol; PL, phospholipid; UN, unidentified; GE, diacylglyceryl ether.
[b]Wet weight basis.

and phospholipid (PL) (22.1%) (Table 1). *Spongiobranchaea australis* has 39.5–66.7% TAG, 0.4–0.8% FFA, and 30.0–58.1% PL. ST are present in all pteropods (0.7–6.7%) along with minor amounts of WE (trace–0.8%) (Table 1).

ST. The major ST in *C. limacina* include 24-methylcholesta-5,24(28)*E*-dien-3β-ol (24-methylenecholesterol, 21.5%), cholesta-5,22*E*-dien-3β-ol (*trans*-dehydrocholesterol, 19.0%), 24-methylcholesta-5,22*E*-dien-3β-ol (brassicasterol, 17%), cholesta-5,24*E*-dien-3β-ol (desmosterol, 15.6%), cholest-5-en-3β-ol (cholesterol, 11.6%), and 24-norcholesta-5,22*E*-dien-3β-ol (24-nordehydrocholesterol, 6.5%) (Table 2). The major ST in *L. helicina* are similar to those in *C. limacina* and include 24-methylenecholesterol (22.8–24.1%), *trans*-dehydrocholesterol (14–16.4%), brassicasterol (15.8–16.8%), desmosterol (17.4–19.7%), cholesterol (9.1–9.8%), and 24-nordehydrocholesterol (7–9.1%). The sterol composition of *S. australis* differs from both *C. limacina* and *L. helicina* in that *S. australis* has less 24-methylenecholesterol (12.7–12.9%), more *trans*-dehydrocholesterol (30.9–34.0%), slightly more brassicasterol (l9.0–20.5%), less desmosterol (4.4–4.5%), slightly less cholesterol (7.7–8.6%), and slightly more 24-nordehydrocholesterol (9.7–9.8%) (Table 2). Five minor ST were also detected: cholesta-5,22*Z*-dien-3β-ol (*cis*-dehydrocholesterol, 1.3–3.2%), 24-ethylcholesta-5,22*E*-dien-3β-ol (stigmasterol, 0.4–1.4%), 24-ethylcholest-5-en-3β-ol (24-ethylcholesterol, 0.2–3.2%), 24-ethylcholesta-5,24(28)*Z*-dien-3β-ol (isofucosterol, 0.9–2.9%), and an unidentified C_{30} ST, containing two double bonds (1.0–2.1%) (Table 2). Five stanols were found in minor amounts in the Southern Ocean pteropods. These include 5α-24-norcholest-22*E*-en-3β-ol (24-nordehydrocholestanol, 0.1–0.6%), 5α-cholest-22*E*-en-3β-ol (dehydrocholestanol, 0.1–0.7%), 5α-cholestan-3β-ol (cholestanol, 0.2–2.2%), 24-methyl-5α-cholest-22*E*-en-3β-ol (brassicastanol, 0.2–0.7%), and 24-methyl-5α-cholest-24(28)*E*-en-3β-ol (24-methylenecholestanol, 0.8–0.9%) (Table 2).

TABLE 2
Sterol Composition of Southern Ocean Pteropods

Sterol	Peak no.[a]	Percentage composition					
		Clione limacina	*Limacina helicina*			*Spongiobranchaea australis*	
24-Nordehydrocholesterol	1	6.52	6.96	9.07	8.58	9.73	9.81
24-Nordehydrocholestanol	2	0.07	—	—	—	0.55	0.58
cis-Dehydrocholesterol	3	1.73	1.65	1.28	1.37	3.19	2.82
trans-Dehydrocholesterol	4	19.05	13.97	16.36	14.90	34.02	30.93
Dehydrocholestanol	5	0.11	—	—	—	0.65	0.65
Cholesterol	6	11.64	9.09	9.79	9.08	8.56	7.72
Cholestanol	7	0.15	0.15	—	—	n.d.[b]	2.16
Desmosterol	8	15.59	19.68	17.42	19.28	4.37	4.49
Brassicasterol	10	17.00	15.82	16.80	15.76	19.03	20.48
Brassicastanol	11	—	0.15	—	—	0.68	0.44
24-Methylenecholesterol	12	21.54	23.61	22.83	24.12	12.93	12.72
24-Methylenecholestanol	13	0.79	0.88	0.75	0.83		
Stigmasterol	14	0.48	1.43	0.44	0.44	0.53	0.36
24-Ethylcholesterol	16	2.20	1.39	1.22	0.20	3.13	3.22
Isofucosterol	18	1.91	2.94	2.54	2.86	0.97	0.87
C_{30}, two double bonds	20	1.01	2.97	1.52	1.75	0.96	1.28
Other		0.22	0.22	—	0.83	0.73	1.4
Total		100.00	100.00	100.00	100.00	100.00	100.00

[a]Peak number on gas chromatogram.
[b]n.d., not determined.

GE. The electron ionization-mass spectra of silylated (1-*O*-alkyl-2,3-di-*O*-TMSi) GE showed the following features: a base peak at 205 *m/z*; ions derived from the glycerol-2,3-di-*O*-TMS moiety, namely, *m/z* 103, 117, 130, 133, and 147; M – 90, M – 104, M – 147, and M – 180. The M^+ was generally not observed. The GC–MS analyses confirmed that the ether lipids of the three Southern Ocean pteropods are a mixture of predominantly saturated with lower levels of monounsaturated 1-*O*-alkyl glyceryl ethers. The principal GE of the three pteropod species is 1-hexa-decylglyceryl ether (16:0); 60% for *C. limacina,* 55% for *S. australis,* and 46% for *L. helicina* (Table 3). The GE 1-penta-decylglyceryl ether (15:0) is second in importance in *C. limacina* (21%), whereas hexadec-9-enylglyceryl ether (16:1) is the second-most abundant GE in *S. australis* (l9%), which has only 4% 15:0 GE. *Clione limacina* has less high molecular weight GE (i18:0–20:1 GE summed are 7%), whereas in *S. australis* these GE (i18:0-20:1) are 18% and in *L. helicina* 22% of the total (Table 3). The GE eicosa-9-enylglyceryl ether (20:1) is 8% and 10% in *S. australis* and *L. helicina,* respectively; however in *C. limacina* it is 3% of the total. Also, the GE octadecylglyceryl ether (18:0) is 4% in *S. australis* and *L. helicina* whereas in *C. limacina* it is 1% of the total (Table 3).

Fatty acids. Clione limacina and *L. helicina* both differ substantially from *S. australis* because they have less eicosapentaenoic acid 20:5n-3 (7.2–11.7%) than *S. australis* (21.5–23.3%) 20:5n-3 (Table 4). The sum of the polyunsaturated fatty acids (PUFA) is therefore greater for *S. australis* (46–48%) than for *C. limacina* (26.7%) and *L. helicina* (32.2–42.8%). Docosahexaenoic acid 22:6n-3 is the second-most common PUFA in *S. australis* (18.7–19.1%) and the major PUFA in both *C. limacina* (15.8%) and *L. helicina* (16.3–21.9%). Although levels of 22:5n-3 are generally low, this PUFA is twice as abundant in *L. helicina* (2.6–2.7%) than in *C. limacina* (l.l%) and *S. australis* (1.1–1.4%). The PUFA 20:2n-6 is also more important in *L. helicina* (3.3–3.8%) than *S. australis* (1.1–1.3%), and it was not detected in *C. limacina* (Table 4).

Clione limacina and *L. helicina* also differ substantially from *S. australis* because they have more of the monoene 20:1n-7*c* (11% for *C. limacina,* 17.8–23.1% for *L. helicina*) than *S. australis* (3.7–3.8%) (Table 4). They also have more 20:1n-9*c* (7.7% for *C. limacina* and 3.5–4.6% for *L. helicina*) than *S. australis* (1.7%). The sum of the monounsaturated fatty acids is therefore greater for *C. limacina* (33.4%) and *L. helicina* (26.3–38.8%) than *S. australis* (19.2–19.3%). Despite this, *S. australis* has considerably more 16:1n-7*c* (6.4–6.5%) than *C. limacina* (2.5%) and *L. helicina* (0.6–3.4%). The two isomers of 18:1n-9*c*, and 18:1n-7*c*, together are 8.6% for *C. limacina*, 3.3–10.6% for *L. helicina*, and 5.5–6.4% for *S. australis*.

The principal saturated fatty acids in *C. limacina* and *L. helicina* are palmitic acid (16:0) and stearic acid (18:0) (Table 4). In *C. limacina*, there is 11.8% of 16:0 and 6.6% of 18:0, whereas in *L. helicina* there is 9.8–16.1% of 16:0 and 5.4–16.8% of 18:0. In *S. australis*, the principal saturated fatty acids are 16:0 (17.8–18.2%), 18:0 (3.4–3.8%), and 14:0 (3.7–3.8%). In *C. limacina*, the saturated fatty acids 17:0 (4.8%) and a17:0 (coeluting with 17:1n-8*c*) (8.9%) are also important. Levels of 17:0 and a17:0 in the other pteropods are uniformly low (0.3–2.2%).

TABLE 3
1-*O*-Alkyl Glycerol Composition of Southern Ocean Pteropods

	Percentage composition		
Ether alkyl chain	*Clione limacina*	*Limacina helicina*	*Spongiobranchaea australis*
14:0	—	8	3
15:0	21	4	4
16:1	3	9	19
16:1	—	4	—
16:0	60	46	55
i17:0	2	—	—
a17:0	3	4	—
17:0	5	3	2
i18:0	<1	—	—
18:1	1	5	6
18:0	1	4	4
i19:0	1	—	—
20:1	trace[a]	3	—
20:1	3	10	8

[a]Trace: less than 0.5%.

DISCUSSION

Occurrence of DAGE in Antarctic zooplankton. The high level of DAGE (28%) in the pteropod *C. limacina* from Antarctic waters (Bransfield Strait) is unusual in marine macrozooplankton (Table 1). Lee (16) reported 19% DAGE in *C. limacina* from Bute Inlet, British Columbia, but the supportive data were unpublished. Published reports on lipid composition of Antarctic invertebrate zooplankton either contain no reference to DAGE or report its presence as less than 1.5%. Hagen (l7) found 1.3% DAGE in *C. limacina* collected from the Antarctische Halbinsel, and DAGE was not reported in pooled samples of *C. limacina* collected from the Weddell Sea in l985. *Limacina helicina* samples had 0–1.5% DAGE (17). From the fatty acid data, it seemed that DAGE constituted 6% in *Clione* specimens (Hagen, W., personal communication). The Thecosome pteropod *Clio pyramidata* had 0.3% DAGE (17). Reinhardt and Van Vleet (5) found TAG, WE, and PL as the major lipid classes in 16 species of Antarctic invertebrate zooplankton, including amphipods, copepods, polychaetes, chaetognaths, cnidarians, tunicates, mysids, and euphausiids as well as mixed plankton. Clarke (l8) found 0.3–1.5% DAGE in the Antarctic isopods *Serolis pagenstecheri* and *S. cornuta*. Fricke *et al.* (l9) found 0.3–0.6% DAGE in Antarctic krill *Euphausia superba*. Hallgren and Stallberg (20) reported less than 1.5% DAGE in benthic invertebrates including marine crayfish, shrimp, and sea mussels (species identifications not given). Some benthic mollusks, such as *Octopus dofleini*, have 5% DAGE (21). The high DAGE percentage in *C. limacina* is accompanied by a

TABLE 4
Fatty Acid Composition of Southern Ocean Pteropods

	Percentage composition					
Fatty acid	*Clione limacina*		*Limacina helicina*		*Spongiobranchaea australis*	
14:0	0.51	1.43	1.11	1.38	3.71	3.84
15:0	1.64	—	0.20	0.19	1.00	1.04
16:0	11.75	16.13	12.53	9.79	18.21	17.83
17:0	4.76	0.76	0.37	0.34	1.13	1.18
18:0	6.58	16.75	6.10	5.38	3.43	3.79
19:0	0.44	—	0.18	0.16	0.06	0.07
20:0	0.82	0.80	2.00	0.35	0.07	0.07
22:0	—	—	0.05	—	0.03	0.03
24:0	—	—	0.21	0.08	—	—
Sum saturates	25.68	35.89	21.58	17.66	27.63	27.84
i14:0	—	—	—	—	0.75	0.92
i15:0	—	—	0.13	0.08	0.08	0.08
i16:0	—	—	0.16	0.08	0.11	0.11
i17:0	0.49	0.50	0.52	0.39	0.49	0.47
a17:0[a]	8.92	1.21	1.64	0.96	2.15	1.82
Sum branched	9.41	1.70	2.45	1.52	3.58	3.40
16:1n-9*c*[b]	—	—	—	—	0.14	0.11
16:1n-7*c*	2.49	—	0.58	3.44	6.43	6.47
16:1n-5*c*	—	—	0.11	0.14	0.15	0.13
18:1n-9*c*	3.11	1.92	1.14	6.81	1.57	1.72
18:1n-7*c*	5.53	2.18	2.12	3.75	4.78	4.75
18:1n-7*t*	—	—	—	—	0.04	—
18:1n-5*c*	0.55	—	0.25	0.28	0.40	0.36
20:1n-11 and n-3*c*	2.63	0.89	—	—	—	—
20:1n-9*c*	7.68	3.50	4.56	4.03	1.76	1.73
20:1n-7*c*	10.98	17.75	23.07	19.94	3.68	3.77
22:1n-9*c*	0.25	0.26	0.28	—	0.14	0.13
22:1n-7*c*	0.22	0.10	0.08	—	0.07	0.05
24:1n-9*c*	—	0.04	0.05	—	0.03	0.02
Sum monounsaturates	33.43	26.25	32.23	38.81	19.19	19.25
18:2n-6[c]	1.08	1.45	0.51	10.06	1.60	1.61
18:4n-3	0.35	—	0.41	0.61	0.78	0.66
20:2n-6	—	3.29	3.80	3.36	1.13	1.26
20:3n-6	—	—	—	—	0.25	0.30
20:4n-3	1.24	1.21	1.66	1.37	0.64	0.65
20:5n-3[c]	7.15	7.26	11.74	11.18	21.45	23.29
22:5n-3	1.11	2.63	2.71	2.55	1.41	1.11
22:6n-3	15.81	16.31	21.94	21.15	18.71	19.07
Sum PUFA	26.74	32.15	42.77	41.30	45.98	47.95
Other	4.74	4.00	0.98	0.71	3.62	1.55
Total	100.00	100.00	100.00	100.00	100.00	100.00

[a]Includes 17:1n-8*c*.
[b]The suffixes *c* and *t* denote *cis* and *trans* geometry, respectively, for monounsaturated fatty acids.
[c]Trace amounts of 18:3n-3 and 20:4n-6 were also present, coeluting with 18:2n-6 and 20:5n-3, respectively. Abbreviation: PUFA, polyunsaturated fatty acids.

high lipid content, 50.8 mg/g wet weight, an order of magnitude greater than in *L. helicina* and *S. australis* (Table 1).

Distribution of DAGE in other marine organisms. DAGE is an abundant component of liver oil of certain shark species (22). Plunket shark (*Centroscymnus plunketi*), Pacific sleeper shark (*Somniosus pacificus*) (23), and the leafscale gulper shark (*Centrophorus squamosus*) (24) have livers rich in DAGE. The liver of many of these sharks is over 50% oil; in some it is over 80% (25). The dogfish, *Squalus acanthias*, has 38–45% DAGE in the liver oil, and the holocephalans *Chimaera barbouri*, *C. ogilbye*, and *Hydrolagus colliei* have more than 50% DAGE in the liver oil (3). *Hydrolagus novaezealandiae* has 66% DAGE in its liver oil (26). Methoxyglyceryl ethers, first isolated from Greenland shark liver lipids, have antibiotic activity and inhibit the growth of tumors in mice (27). DAGE is also an abundant component of

certain deep sea squids. The liver and stomach contents of *Moroteuthis robusta* (giant squid, family Onychoteuthidae) and the liver of Gonatid squids (family Gonatidae, *Berryteuthis magister*, *Gonatopsis makko*, and *G. borealis*) also contain significant amounts of DAGE (28–30).

GE composition. The GE composition of three Southern Ocean pteropods (Table 3) is different from deep-sea sharks and squids. Pteropod GE are dominated by 1-hexadecylglyceryl ether (16:0; 46–60%), whereas the major GE in deep-sea sharks and marine DAGE in general is octadec-9-enylglyceryl ether 18:1n-9*c* (42–62%) (23,31). Although deep-sea squid *M. robusta* has less 18:1n-9*c* GE (24–26%) (30), it is much more than the level of 18:1 GE in pteropods (1–6%, Table 3). In addition, the pteropod *C. limacina* has 21% 1-penta-decylglyceryl ether (15:0 GE) which was generally not reported in deep-sea sharks and is only present at 1–1.4% levels in deep-sea squids. At the present time, the reasons for the high relative proportion of the C_{15} GE are not known. Two isomers of 20:1 GE were also observed (Table 3). This observation is similar to the fatty acid profile where two isomers 20:1n-7*c* and 20:1n-9 were also observed. The similarity in profiles is consistent with a common origin. In *C. limacina* in particular, the GE composition is dominated by lower molecular weight GE than in the deep-sea sharks and squids. The GE of *C. limacina* are also different from *L. helicina* and *S. australis* which are characterized by somewhat higher hexadec-7-enylglyceryl ether (16:1n-7*c*; 9–19%) levels than *C. limacina* (3% 16:1n-7*c*) and higher levels of i18:0–20:1 (summed) (22–18%, respectively) (Table 3).

Two isomers of 20:1 were also observed in the alkyl GE diol fraction of *L. helicina*. The double-bond position was not determined for the GE fractions; however, based on the fatty acid profiles, the two alkyl GE chains are presumed to be the n-7 and n-9 isomers. In comparison, only one main isomer was present in *Clione* and *Spongiobranchaea* with trace levels of a second isomer also present; based on retention time data, the main component was presumed to be the 20:1n-7 isomer. This observation is in contrast to the fatty acid profiles where two isomers (20:1n-7*c* and 20:1n-9*c*) were present in all three animals. In addition for *Clione* and *Spongiobranchaea*, the n-7/n-9 ratio for the fatty acids was generally between 1–2. Although the n-7 20:1 isomer was present at higher relative levels than the n-9 isomer in the fatty acid fraction, the ratio of n-7/n-9 for GE was significantly greater than observed for the fatty acid fractions in both *Clione* and *Spongiobranchaea*. We presently can offer no explanation for these ratio differences between lipid classes (GE from DAGE vs. fatty acids from all lipids) in the proportion of isomers. Kang *et al.* (31) have noted similar ratio differences for DAGE GE and total lipid fatty acids from both shark liver oil rich in DAGE fed to Atlantic salmon and for DAGE and total lipid recovered from the salmon feces.

Comparative lipid compositional features. The PL and ST reported in Table 1 probably include membrane structural PL and membrane ST. The relatively higher amounts of PL in *L. helicina* (34.2–39.4%) vs. *C. limacina* (22.1%) are consistent with their lower lipid (2.4–6.9 mg/g wet weight). The same feature is observed for *S. australis* (3.3–4.8 mg/g wet weight). Also *L. helicina* and *S. australis* have trace or low DAGE (Table 1). The high FFA in *L. helicina* (54.9–59.1%) reflect the time necessary for sorting and counting in a heated zooplankton van prior to freezing. The small body size of *L. helicina* compared with *C. limacina* means that lipase enzyme breakdown of TAG (not detectable in *L. helicina*, Table 1) and/or PL occurred more rapidly than in the larger *C. limacina* (23.9% FFA). We have no explanation for the high PL values in *L. helicina* (Table 1). An alternative explanation might be that the FFA are used as an energy store. The high TAG (39.5–66.7%) in *S. australis* reflect a lack of lipase activity because *S. australis* was sorted and counted in a laboratory aboard the R/V *Aurora Australis* maintained at −1°C. Freezing was also accomplished more rapidly after capture and was in liquid nitrogen. The presence of WE (trace to 0.8%) in all pteropods (Table 1) may mean that these WE were obtained from the food chain. Copepods are rich sources of WE in polar waters (1) and may be the dietary source of WE in pteropods.

The similar ST composition of *C. limacina* and *L. helicina* (Table 2) reflects the feeding selectivity of *C. limacina*, which is believed to feed exclusively on *L. helicina* (9). The ST of *S. australis* differ from *L. helicina* and *C. limacina* (i.e., less 24-methylenecholesterol and more *trans*-dehydrocholesterol, Table 2) because *S. australis* feeds exclusively on certain *Clio* species, such as *C. pyramidata* (17). Both *Limacina* and *Clio* are herbivorous Thecosome pteropods, and their ST are derived from dietary phytoplankton. Desmosterol, for example, in *L. helicina* (17.42–19.68%, Table 3) is probably derived from dealkylation of dietary phytosterols. Desmosterol may also be of some importance in providing cholesterol, since it is an intermediate in cholesterol synthesis. Gastropods can synthesize cholesterol *de novo* (32), but it is a very slow process, as evidenced by the low cholesterol levels in these pteropods (11.6% cholesterol in *C. limacina*, Table 2). Cholesterol was present in only six of 14 diatom species studied by Barrett *et al.* (33). The ST 24-methylenecholesterol (21.5–24.1% in *C. limacina* and *L. helicina*) is the main ST in the diatom *Chaetoceros* (34). Brassicasterol is a major ST in some prymnesiophytes such as *Phaeocystis*, and it is important in *L. helicina* (15.8–16.8%, Table 2). The phytoplankton composition of the Bransfield Strait waters where *C. limacina* and *L. helicina* were collected was characterized by microplanktonic diatoms, such as *Chaetoceros*, *Nitzschia*, and *Rhizoselenia*, as well as 2–5-nm flagellates during l990–l993 (35). *Rhizoselenia setigera* was reported to have 94.2% desmosterol and *Nitzschia closterium* 100% desmosterol (33). The area where *S. australis* was collected is characterized by a different phytoplankton assemblage, which may be reflected in its different ST composition compared to that in the other two pteropods (Table 2).

The similar fatty acid composition of *C. limacina* and *L. helicina* bears out the feeding selectivity already discussed. Despite this, there are differences in the percentage composi-

tion of a17:0/17:1n-8*c* which are unexplained (Table 4). *Spongiobranchaea australis* fatty acids are different from those of *C. limacina* and *L. helicina* in that *S. australis* has over twice as much eicosapentaenoic acid 20:5n-3 (Table 4). This may indicate greater availability of this n-3 PUFA in the phytoplankton grazed by *Clio pyramidata*, the food of *S. australis*. The 20:5n-3 PUFA is found in the diatoms *Chaetoceros calcitrans*, *Skeletonema costatum*, and *Thallassiosira pseudonana*, where it is 4.6–11.1% of the total fatty acids (36). *Clione limacina* and *L. helicina* are enriched in the monoenes 20:1n-7*c* and 20:1n-9*c* in contrast to *S. australis*. This also emphasizes the different sources of food available to these two groups of pteropods.

Potential uses of DAGE. DAGE may be important in the treatment of hematopoiesis and radiation sickness (37,38). Glyceryl ethers are useful as surfactants in cosmetics and ointments, and ratfish liver DAGE are intermediates in the synthesis of alkylacetoglycerophosphocholine, a platelet-activating factor (29,39). Methoxy-glyceryl ethers have been reported in shark liver oil (20) and have antibiotic activity and inhibit the growth of tumors in mice (27). It has been stated that the glyceryl ethers, including those of chimyl or batyl alcohol (or their monounsaturated derivatives), have enormous economic potential because they possess this range of healing activity (40). Shark ether lipids can become a good source of ether-linked lipids, which increase membrane plasmalogen levels in patients suffering from the genetic Zellwegere cerebrohepatorenal syndrome (41). It would be interesting to confirm that the pteropod DAGE also have anticarcinogenic activity, since pteropod culture could be a rapid and renewable source of DAGE. In contrast, the presently utilized deep-sea shark and ratfish may be showing signs of overfishing in selected locations. Harvest of pteropods therefore might be an alternative source of DAGE. *Clione limacina* amounts to 80% of the total Gymnosome population in the north Atlantic Ocean and is so common that it is a food of the Greenland Right whale and some fishes (9). It reaches 50–70 mm in length in cold waters. Further analyses of *C. limacina* and other pteropods from a range of locations are recommended and will be pursued. The economics for harvesting *Clione* presently may appear unattractive. However, culture of this species, including as a possible direct source of DAGE or for use as a source of transgenic material for further application, may have better potential.

A possible buoyancy role for DAGE. A convincing argument for buoyancy regulation as a function for DAGE was made by Malins and Barone (42), who found the concentration ratios of DAGE to TAG to significantly increase in livers of weighted *Squalus acanthias* when compared to an unweighted control group. DAGE gives 23% more lift than TAG in 1.025 g/cc sea water (4). The change was not due to selective mobilization of TAG but involved a net increase in DAGE. Malins and Barone (42) suggested that *S. acanthias* regulates buoyancy by the selective metabolism of DAGE and TAG during vertical migrations, and that this may substitute for the swimbladder in teleost fishes that is not found in sharks.

We propose a buoyancy function for the 28% DAGE found in *C. limacina*. Most lipids are less dense than seawater, and therefore increase the overall buoyancy of aquatic organisms, but their main functions are energy storage and as structural (membrane) components (43). Lee (l), for example, starved *Clione* from Canadian waters for 3 mon and observed a loss of 85% of their lipid (mostly TAG). Significant structural differences between *Clione* and *Limacina*, however, support a buoyancy function. *Limacina* is a Thecosome pteropod, with a calcareous test (shell), but the density of the test is offset by bubbles around it. *Limacina* also has long floppy swimming appendages, like *Clio pyramidata*, another Thecosome (shelled) pteropod (44). These swimming appendages help these pteropods maintain their position in the water column, and the increased surface area slows down sinking. In contrast, both *C. limacina* and *S. australis* are Gymnosome (naked) pteropods, lack bubbles, and have compact, hard bodies, with short, stubby swimming appendages. Both *Clione* and *Spongiobranchaea* have DAGE (28% and 0.9–1.7%, respectively, Table 1), whereas *Limacina* has only trace amounts of DAGE. *Clione* is lipid-rich, whereas *Limacina* is low in lipid (17) (Table 1). Since *S. australis* has both low lipid and low DAGE, it may use an alternative strategy for buoyancy. The Gymnosome pteropods selectively feed on the herbivores *Limacina* and *Clio*, so it is certainly to their advantage to remain at the same level in the water column as their prey. DAGE may give just the added uplift to accomplish the appropriate buoyancy for this selective feeding in *C. limacina*.

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Sulfoquinovosyl Diacylglycerols from the Alga *Heterosigma carterae*

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ABSTRACT: An extract of the chloromonad *Heterosigma carterae* (Raphidophyceae), cultivated in natural seawater, contained a complex mixture of sulfoquinovosyl diacylglycerols. Palmitoyl (16:0), three isomers of hexadecenoyl (16:1 *cis* Δ9, Δ11, Δ13), and eicosapentenoyl (20:5) were found to be the main fatty acyl substituents. Exact double-bond sites were determined by mass spectrometry analysis of the corresponding nicotinyl derivatives. Four major sulfoquinovosyl diacylglycerol components were partially purified and identified as **1**–**4** by interpretation of their nuclear magnetic resonance and mass spectral data. In addition, complete analysis of the *H. carterae* sulfoquinovosyl diacylglycerols was performed using high-performance liquid chromatography combined with electrospray tandem mass spectrometry.

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Blooms of the microalga *Heterosigma carterae* (formerly *H. akashiwo*) (1) have been implicated in massive fish mortalities at aquaculture sites along the North American Pacific Coast (2) and also in some other regions of the world, particularly Chile (3), Japan (4), New Zealand (5), and Spain (6). Biochemical and biological studies indicated that *H. carterae* produced a toxic substance responsible for the fish deaths (1), and this prompted us to investigate the molecular structures of the sulfoquinovosyl diaclglycerol (SQDG) components produced at high levels by this organism (7,8). SQDG components isolated from other organisms have been shown to exhibit potent antiviral activity, particularly against the human immunodeficiency virus (HIV-1) (9), and also antitumor-promoting activities (10). Furthermore, glycolipids have been implicated in fish mortalities caused by *Chrysochromulina polylepis* (11).

In practice, the isolation of pure SQDG from the complex mixtures usually encountered in nature, which differ only in the length and/or degree of unsaturation of their fatty acyl substituents, is extremely difficult (12). Furthermore, a practical and rapid method for the structural analysis of these substances has been elusive. The goal of the present study was therefore to develop a new method for the structural elucidation of the SQDG produced by *H. carterae*, utilizing liquid chromatography combined with electrospray tandem mass spectrometry (LC/MS/MS). As the structure of the sulfoglycoglycerol moiety is essentially the same for all SQDG isolated and identified to date [i.e., 3-*0*-(6-sulfo-α-D-quinovopyranosyl)-*sn*-glycerol] (13), the objective was to focus on determination of the structures and positional assignments (*sn*-1 or *sn*-2) of the fatty acyl substituents. Nuclear magnetic resonance (NMR) spectroscopy was used to confirm the structure of the sulfoquinovosyl glycerol moiety, and also to firmly establish the structures of purified *H. carterae* SQDG identified using the LC/MS/MS technique.

RESULTS AND DISCUSSION

The SQDG fraction was obtained from the chloroform/methanol extract of *H. carterae* cells using silica gel column chromatography. In order to minimize air oxidation of any polyunsaturated fatty acyl moieties present in the SQDG fraction, the extraction and lipid fractionation were completed within 1 d, and fractions were stored under nitrogen, at –80°C, until further use. Analysis by ^{1}H and ^{13}C NMR spectroscopy and by thin-layer chromatography showed the SQDG fraction to be free of other glycolipids.

Identification of the fatty acyl substituents of H. carterae *SQDG*. A portion of the *H. carterae* SQDG fraction was reacted with boron trifluoride in methanol to liberate the fatty acyl substituents as their methyl ester derivatives, which were analyzed by gas chromatography/mass spectrometry (GC/MS). The major fatty acid methyl esters (FAME) were identified as 14:0, 16:0, 20:5, and three isomers of 16:1 (see Table 1). Minor components were determined to be 15:0, 18:3, and 18:4 (Table 1). By comparison of the GC/MS data with those obtained for FAME standards, the structure of one of the 16:1 isomers was

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Abbreviations: AQ, acquisition time; COSY, correlated spectroscopy; EI, electron impact; FA, fatty acid; FAME, fatty acid methyl ester; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum correlation; LC, liquid chromatography; LC/MS/MS, combined liquid chromatography/tandem mass spectrometry; LSIMS, liquid secondary ion mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; SQDG, sulfoquinovosyl diacylglycerol; SQMG, sulfoquinovosyl monoacylglycerol; SW, spectral width; TOCSY, total correlated spectroscopy.

TABLE 1
Fatty Acid Composition of the SQDG Isolated from *Heterosigma carterae*[a]

Fatty acid	R_t (min)	Amount (%)[b]
14:0 *n*	7.0	6.0
15:0 *n*	7.5	1.4
16:0 *n*	8.2	24.0
16:1 Δ9 *cis*	8.4	7.7
16:1 Δ11 *cis*	8.5	11.8
16:1 Δ13 *cis*	8.6	31.2
18:3 Δ9, Δ12, Δ15 all-*cis*	11.3	2.6
18:4 Δ6, Δ9, Δ12, Δ15	11.6	2.7
20:5 Δ5, Δ8, Δ11, Δ14, Δ17 all-*cis*	14.6	12.7

[a]Abbreviations: SQDG, sulfoquinovosyl diacylglycerol; R_t, retention time.
[b]Amounts were estimated from peak areas in the gas chromatography/mass spectrometry total ion current chromatograms for the fatty acid methyl esters.

determined to be the methyl ester of palmitoleic acid (16:1 *cis*-Δ9), and the 14:0, 16:0, 18:3, and 20:5 FAME were identified as the methyl esters of myristic, palmitic, linolenic (*cis*-Δ9, Δ12, Δ15), and eicosapentaenoic (*cis*-Δ5, Δ8, Δ11, Δ14, Δ17) acids, respectively. The position and stereochemistry of the carbon-carbon double bonds present in the remaining two 16:1, and the 18:4 FAME could not be determined by comparison of the GC/MS data with available standards. [After this work was completed, it came to our attention that the 18:4 (all-*cis* Δ6, Δ9, Δ12, Δ15) FAME is now available from Sigma Chemical Company (St. Louis, MO).]

To determine the position of the double bonds in the unidentified 16:1 and 18:4 SQDG fatty acyl groups and to confirm the assignments previously made using FAME standards analyzed by GC/MS, a portion of the *H. carterae* SQDG fraction was reacted with lithium aluminum hydride to liberate the fatty acyl moieties as the corresponding alcohols. The alcohols were then converted to their nicotinate derivatives, and the resulting mixture was analyzed by GC/MS. Interpretation of the mass spectra of the nicotinate derivatives (discussed shortly) allowed assignment and confirmation of all the carbon-carbon double-bond sites (Table 1). The two unknown SQDG hexadecenoyl moieties were identified as 11-hexadecenoyl (16:1 Δ11) and as 13-hexadecenoyl (16:1 Δ13) (7), and the 18:4 SQDG fatty acyl group was determined to be 18:4 (Δ6, Δ9, Δ12, Δ15). Additionally, the 15:0 fatty acyl substituent was found only as the saturated species.

Previous investigations have reported the use of picolinyl ester derivatives of fatty acids (FA) in order to establish the carbon-carbon double-bond positions (14–18). However, the use of picolinyl ester or nicotinate derivatives for determination of double-bond positions in SQDG fatty acyl moieties has not yet been described. For SQDG, three steps would be required for preparation of the picolinyl esters: (i) cleavage of the SQDG to liberate the FA, (ii) formation of the FA chloride using thionyl chloride, and (iii) esterification with pyridylcarbinol. Steps i and ii involve harsh conditions for polyunsaturated FA prone to air oxidation, although a modification has recently been described for step ii (17,18). The overall reaction procedure described here for the formation of the nicotinate derivatives from the SQDG fatty acyl substituents proceeds in good yield with minimal side reactions, and only two steps are required. The preparation of nicotinate derivatives of long-chain alcohols and their analysis by electron impact mass spectrometry (EI/MS) was previously described by Vetter and Meister (19) and by Harvey (16). However, in contrast to a previous report (16), we have found that the formation of the nicotinate derivatives from long-chain alcohols is highly reproducible when using nicotinyl chloride directly as the reactant in anhydrous pyridine.

To determine the extent of decomposition of the polyunsaturated fatty acyl moieties, the synthesis of the nicotinate derivatives from the SQDG fatty acyl groups was monitored by ^{1}H NMR spectroscopy. A comparison was made between the NMR spectra of the initial SQDG mixture and those of both the long-chain alcohol intermediates and nicotinate products. It was found that there is a stable ratio between the integrals for the protons resonating at *ca.* δ 5.3 ppm (olefinic protons), *ca.* δ 2.8 ppm (doubly allylic methylene protons characteristic of polyunsaturated FA), and the signals in the range between δ 0.7 and δ 2.0 ppm (protons of the aliphatic methylene and terminal methyl groups). This result indicated that the polyunsaturated SQDG fatty acyl moieties were not degraded to a detectable amount more than were the saturated fatty acyl groups, an important objective of this study.

The EI mass spectra of the nicotinate derivatives of the saturated 14:0, 15:0, and 16:0 SQDG fatty acyl moieties show a regular series of fragment ions down to *m/z* 178, resulting from charge-site remote radical-induced cleavage along the hydrocarbon chain. Figure 1 (A and B) shows the EI mass spectra of the nicotinate derivatives of two of the three isomeric SQDG hexadecenoyl (16:1) acyl groups. These two background-subtracted mass spectra (as well as that for the 16:1 Δ9 isomer, not shown) were taken from a single GC/MS experiment, at the crests of GC peaks eluting within a 30-s period. Each 16:1 nicotinate derivative exhibited a molecular ion at *m/z* 345, but the series of fragment ions between *m/z* 345 and *m/z* 178 showed marked differences, in terms of both the *m/z* values and the relative intensities of the fragment ions observed. As described by Vetter and Meister (19), a minimum in the relative intensities of the fragment ions for the nicotinate derivatives of monounsaturated fatty alcohols denotes the position of the double bond. Straightforward interpretation of the mass spectra of the nicotinate derivatives allowed assignment of two of the 16:1 SQDG fatty acyl groups as being 11-hexadecenoyl (Fig. 1A) and 9-hexadecenoyl (not shown). The latter assignment is also consistent with a comparison of the GC retention time of the corresponding FAME (determined in a GC/MS experiment) to that of a 16:1 Δ9 standard. The assignment of the final 16:1 SQDG fatty acyl moiety as 13-hexadecenoyl was not possible by simple interpretation of the mass spectrum of its nicotinate derivative (Fig. 1B). In fact, the mass spectral data could easily be misinterpreted as arising from the nicotinate derivative of a 12-hexadecenoyl moiety. However, we have studied the *H. carterae* SQDG 13-hexadecenoyl moiety in detail (7) and

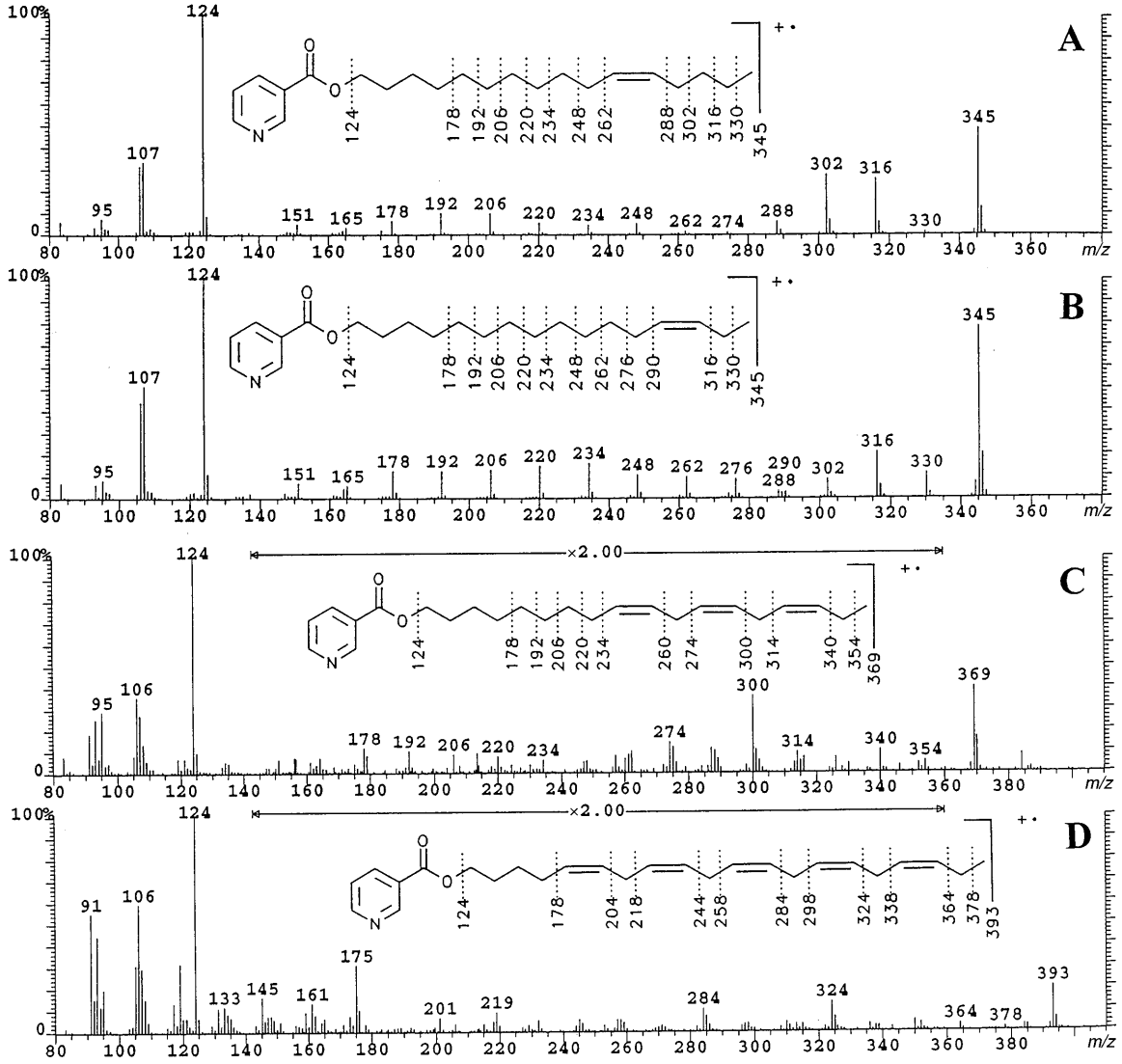


FIG. 1. The gas chromatography/electron impact mass spectra of the nicotinate derivatives of the polyunsaturated sulfoquinovosyl diacylglycerol fatty acyl moieties. A) 16:1 Δ11, B) 16:1 Δ13, C) 18:3 Δ9, Δ12, Δ15, D) 20:5 Δ5, Δ8, Δ11, Δ14, Δ17.

have shown that its nicotinate derivative gives unexpectedly high relative intensities for the fragment ions observed at *m/z* 316 and 302, as is seen in Figure 1B, which is consistent with the observed fragmentation of the picolinyl ester of the homologous 15-octadecenoyl compound (18). These results were in contrast to previous investigations of the total FA-fraction of *H. carterae*, where FAME were used exclusively for structural elucidation of the FA residues (20).

Figure 1 (C and D) shows the EI mass spectra of the nicotinate derivatives of the polyunsaturated *H. carterae* SQDG fatty acyl groups. The signal-to-noise ratio evident in these spectra is much lower than that achievable with more abundant components or with standards, although such a result may be realistic for many minor polyunsaturated components of interest. Furthermore, fragmentations arising from the charge-site remote radical-induced cleavage are seen to be much less intense and increasingly ambiguous with increasing degree of unsaturation. Additionally, the polyunsaturated nicotinate derivatives eluted at higher column temperatures than the saturated or monounsaturated derivatives so that a higher level of background ions was observed. This background noise may not be completely eliminated by background subtraction (e.g., *m/z* 384 in Fig. 1C). The assignments of the 18:3 and 20:5 polyunsaturated SQDG fatty acyl moieties obtained by GC/MS of the FAME were confirmed by the EI mass spectra of their nicotinate derivatives (Fig. 1C and 1D, respectively). The spectrum of the 18:3 nicotinate shows fragmentation similar to that observed by Harvey (15) for the picolinyl ester derivative of *cis* Δ9, Δ12, Δ15-octadecatrienoic acid. In the present case, the dominant signal at *m/z* 300 (Fig. 1C) can be explained by the loss of an allylic radical. The corresponding ion for 18:4 is seen at *m/z* 298 while that for 20:5 is at *m/z* 324 (Fig. 1D), consistent with the assigned structures. However, for 18:4 the signal-to-noise ratio

is too low to allow confirmation by the presence of the expected fragment ions at *m/z* 178 and 192 that the additional unsaturation is indeed located at C-6. It can be seen from Figure 1 that, for the nicotinate derivatives of saturated and monounsaturated fatty alcohols, an ion containing a C-4 chain at *m/z* 178 is generally observed. It was found that for derivatives of polyunsaturated SQDG fatty acyl moieties an ion at *m/z* 178 is seen in the case of 18:3 but is weak for 18:4 and 20:5, consistent with the presence of the proximal double bond at C-5 or C-6.

To assign the configuration of the carbon-carbon double bonds in the 11- and 13-hexadecenoyl SQDG fatty acyl substituents, the purified (7) methyl ester derivatives were examined by ^{1}H NMR spectroscopy. For the methyl ester derivative of the 13-hexadecenoyl substituent, decoupling by irradiation of the allylic methylene protons at δ 2.05 ppm collapsed the multiplet resonating at δ 5.32 ppm to a doublet of doublets with a coupling constant of 10.8 Hz, allowing assignment of the *cis* (*Z*) configuration (21) for the carbon-carbon double bond (Table 2). In the same experiment, the triplet resonance at 0.95 ppm, assigned to the terminal methyl group, collapsed to a singlet. This result proved that the carbon-carbon double bond was in the n-3 (Δ13) position (7). By using the same decoupling technique, the methyl ester derivative of the 11-hexadecenoyl substituent also gave a doublet of doublets for the olefinic protons, with a coupling constant of 10.9 Hz. Thus, the configuration of the double bond in the 11-hexadecenoyl substituent was also assigned as *cis* (*Z*).

The purified methyl ester derivative of the 9-hexadecenoyl substituent was found to be in the *cis* (*Z*) configuration based on GC/MS comparison with an authentic standard. This derivative gave, upon irradiation at 2.05 ppm, a singlet resonance at 5.34 ppm for the two olefinic protons. NMR examination of *cis* and *trans* isomers of the methyl esters of four Δ9 monounsaturated FA standards performed under the same decoupling conditions as described above showed that the *cis* isomers gave a singlet resonance at 5.34 ppm for the olefinic protons, whereas for the *trans* isomers the singlet was observed at 5.38 ppm (Table 2). This result confirmed that the 9-hexadecenoyl fatty acyl substituent was the *cis* (*Z*) isomer.

^{13}C NMR analysis of the methyl ester derivatives of the isolated 9-, 11-, and 13-hexadecenoyl moieties also supported the stereochemical assignments (Table 3). For the *cis* and *trans* standards of 16:1 Δ9, significant differences were observed for both the chemical shifts of the olefinic carbons (*cis*: 130.8 and 130.9 ppm; *trans*: 131.5 and 131.6 ppm) and for the corresponding signals of the adjacent methylene carbons (*cis*: 28.1 and 28.2 ppm; *trans* 33.7 and 33.8 ppm). The ^{13}C NMR chemical shifts assigned to the allylic methylene carbons in both the 16:1 Δ11 and 16:1 Δ13 fatty acyl substituents (Table 3) supported the *cis* stereochemical assignment for the double bonds and are in good agreement with published data (22–24).

Investigation of the intact SQDG from H. carterae *by LC/MS and LC/MS/MS*. With the analysis of the fatty acyl substituents completed, the next step was to examine the intact *H. carterae* SQDG in order to locate the acyl substituents on the sulfoquinovosyl glycerol backbone. Figure 2 shows the flow injection electrospray mass spectrum of the *H. carterae* SQDG in the negative-ion mode. The peaks at *m/z* 837, 813, 791, and 763, which represent singly deprotonated molecules $[M - H]^-$, are the most prominent. On the basis of tandem mass spectrometry (MS/MS) experiments, these ions could be tentatively assigned as SQDG with the following fatty acyl pairings (as described in the following discussion); 20:5/16:1 and/or 18:3/18:3 (*m/z* 837); 18:3/16:1 (*m/z* 813); 16:0/16:1 (*m/z* 791); and 14:0/16:1 (*m/z* 763). On the basis of the fragmentation patterns in their MS/MS spectra, the peaks at *m/z* 823 and *m/z* 869 in Figure 2 were determined to represent peroxidized forms of the two most abundant SQDG (see Table 4) having the 16:0/16:1 and 20:5/16:1 fatty acyl pairings, respectively.

Although it was possible to analyze the major *H. carterae* SQDG by flow-injection MS/MS without chromatographic separation, it was necessary to use LC/MS and LC/MS/MS

TABLE 2
Chemical Shifts of Olefinic Protons of Different Unsaturated Fatty Acid Methyl Esters (FAME) in CD_3OD and Olefinic H-H Coupling Constants Measured After Irradiation at δ 2.05

FAME				
13-Hexadecenoyl methyl ester	16:1	n-3	Δ13	5.30/5.34 *J* = 10.8 Hz
11-Hexadecenoyl methyl ester	16:1	n-5	Δ11	5.33/5.36 *J* = 10.9 Hz
9-Hexadecenoyl methyl ester	16:1	n-7	Δ9	5.34[a]
Palmitoleic acid (*cis*)	16:1	n-7	Δ9	5.34 *s*
Palmitelaidic acid (*trans*)	16:1	n-7	Δ9	5.38 *s*
Oleic acid (*cis*)	18:1	n-9	Δ9	5.34 *s*
Elaidic acid (*trans*)	18:1	n-9	Δ9	5.38 *s*

[a]Signals for both olefinic protons are identical. Abbreviation: *s*, singlet.

TABLE 3
Carbon Nuclear Magnetic Resonance Shifts of Fatty Acid Methyl Esters in CD_3OD

	Synthetic		Isolated		
Position	16:1 Δ9 *cis*	16:1 Δ9 *trans*	16:1 Δ13	16:1 Δ11	16:1 Δ9
Methyl	52.0	52.0	52.0	52.0	52.0
1	175.9	175.6	175.9	175.9	175.9
2	34.8	34.9	34.8	34.8	34.8
3	26.1	26.1	26.0	26.0	26.0
4	30	30	30	30	30
5	30	30	30	30	30
6	30	30	30	30	30
7	30	30	30	30	30
8	**28.1**[a]	**33.7**	30	30	28.0
9	**130.8**	**131.5**	30	30	130.8
10	**130.9**	**131.6**	30	28.1	130.8
11	**28.2**	**33.8**	30	130.6	28.1
12	31	31	28.0	131.0	30
13	31	31	130.2	27.9	31
14	33.0	33.0	132.5	30	33.2
15	23.8	23.9	21.4	24.0	23.8
16	14.5	14.5	14.8	14.1	14.3

[a]Characteristic chemical shifts for *cis* and *trans* isomers are in boldface type.

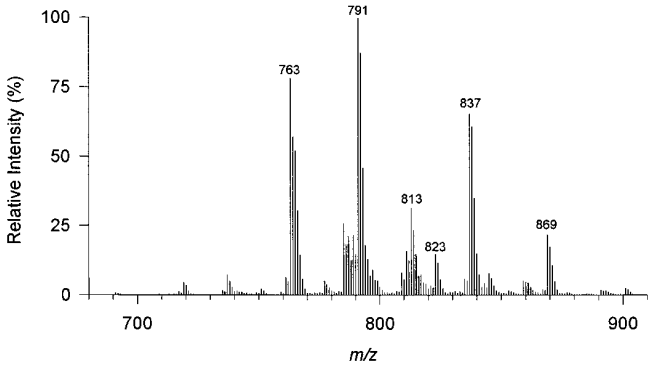


FIG. 2. Flow injection negative ion electrospray mass spectrum of the *Heterosigma carterae* sulfoquinovosyl diacylglycerols.

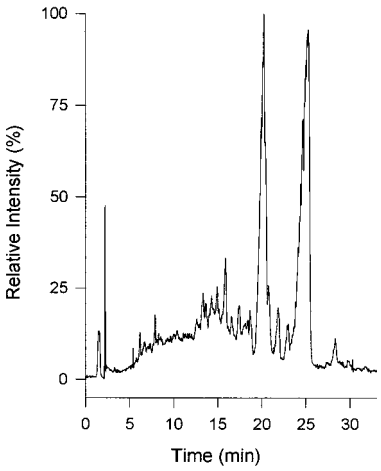


FIG. 3. Total ion current liquid chromatography/mass spectrometry chromatogram for the *Heterosigma carterae* sulfoquinovosyl diacylglycerols.

methods to resolve isomeric and closely related components as well as some minor SQDG components found in this fraction. A separation method employing a basic mobile phase and an alkali-resistant octadecylsilyl stationary phase provided the best separation of the *H. carterae* SQDG (Fig. 3). As demonstrated by the extracted ion chromatograms (Fig. 4) taken from the LC/MS total ion chromatogram shown in Figure 3, closely related SQDG could be adequately resolved.

Analysis of the MS data showed that the two major peaks in Figure 4B corresponded to two SQDG differing by two mass units. The peak at 22.9 min corresponds to an SQDG of mass 766 ($[M - H]^-$ *m/z* 765, 16:0/14:0 fatty acyl pair), whereas the peak at 20.4 min corresponds to the second isotope peak (i.e., two mass units higher than the nominal mass) of the SQDG of nominal mass 764 ($[M - H]^-$ *m/z* 763, 14:0/16:1 fatty acyl pair, see Fig. 4A). This second isotope peak arises owing to the natural abundance of ^{13}C and ^{34}S resulting in an ion at *m/z* 765 for the 14:0/16:1 fatty acyl pair at 17% abundance relative to the all ^{12}C and ^{32}S ion at *m/z* 763. Similarly, the peaks at 25.1 and 28.3 min in Figure 4F correspond to SQDG of mass 792 ($[M - H]^-$ *m/z* 791, 16:0/16:1 fatty acyl pair) and 794 ($[M - H]^-$ *m/z* 793, 16:0/16:0 fatty acyl pair), and the peaks at 20.2 and 20.9 min in Figure 4I correspond to SQDG of mass 838 ($[M - H]^-$ *m/z* 837, 20:5/16:1 fatty acyl pair) and 840 ($[M - H]^-$ *m/z* 839, 20:5/16:0 fatty acyl pair). In each of these cases, the SQDG components differing by two mass units were nearly baseline resolved, and MS/MS experiments could be performed without interferences due to ^{13}C and ^{34}S isotopic variants of a related SQDG of similar mass (see Table 4 for a complete summary of the LC/MS/MS results).

The MS/MS spectrum of the major SQDG (16:0/16:1 fatty acyl pairing) found in the *H. carterae* cell extract, taken from an LC/MS/MS run, is shown in Figure 5. The observed fragmentation pattern is significantly different from that previously observed by fast atom bombardment ionization-MS/MS of SQDG (25). Of particular note is the absence of fragment ions resulting from cleavages along the fatty acyl chains which only arise at higher collision energies than are accessible on the triple quadrupole mass spectrometer used for this experiment. Also, in contrast to the earlier fast atom bombardment ionization-MS/MS results, the most abundant high

TABLE 4
SQDG Composition of *Heterosigma carterae*

FA composition		LC/MS		LC/MS/MS		
sn-1	*sn*-2	R_t (min)	%[a]	M_r	$[M - H - R_{sn\text{-}1}CO_2H]^-$	$[M - H - R_{sn\text{-}2}CO_2H]^-$
14:0	16:1	20.4	8.1	764	535	509
16:0	14:0	22.9	3.0	766	509	537
15:0	16:1	21.8	2.8	778	523	535
16:1	16:1	20.7	2.4	790	535	535
16:0	16:1	25.1	53.0	792	535	537
16:0	16:0	28.3	0.6	794	537	537
18:3	16:1	20.3	3.7	814	535	559
18:3	18:3	19.4	0.2	838	559	559
20:5	16:1	20.2	21.8	838	535	583
20:5	16:0	20.9	0.8	840	537	583
	Others[b]		3.6			

[a]Amounts estimated from LC/MS peak areas.

[b]SQDG in smaller amounts (e.g., pairings with the 18:4 fatty acid). Abbreviations: FA, fatty acid; LC/MS, liquid chromatography/mass spectrometry; LC/MS/MS, combined liquid chromatography/tandem mass spectrometry. For other abbreviations see Table 1.

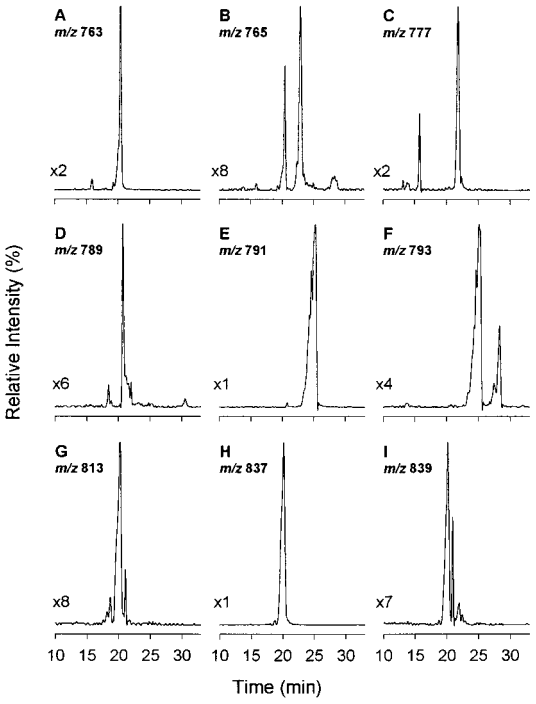


FIG. 4. Extracted ion chromatograms reconstructed from the full-scan liquid chromatography/mass spectrometry data shown in Figure 3 for the *Heterosigma carterae* sulfoquinovosyl diacylglycerols.

mass fragment ions (at *m/z* 537 and *m/z* 535) can be rationalized as arising from a fragmentation involving loss of one acyl substituent from the molecular ion ($[M - H - C_{16}H_{30}0_2]^-$ and $[M - H - C_{16}H_{32}0_2]^-$) and corresponding to fragment ions **b** and **e** shown in Scheme 1. In this Scheme, the fragmentation of SQDG under MS/MS conditions is shown. The fragments **b–e** may be used for determination of the FA substitution. Fragment ions at *m/z* 553 and *m/z* 555 arising from the loss of a ketene analog ($[M - H - R_1C{=}0]^-$ and $[M - H - R_2C{=}0]^-$ (25), ions **c** and **d** in Scheme 1) are of very low intensity in the LC/MS/MS spectrum. Peaks assigned as the fragment ions corresponding to the free FA ($[C_{16}H_{31}0_2]^-$ and $[C_{16}H_{29}0_2]^-$ are clearly visible at *m/z* 255 and *m/z* 253. Other peaks appearing in Figure 5 could be readily assigned to fragment ions **f–o** which can be considered characteristic for the substance class of SQDG under MS/MS conditions, independent of their FA substitution pattern (Scheme 2) (25).

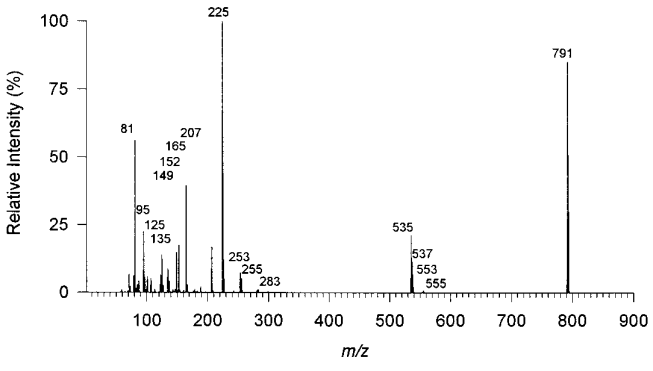
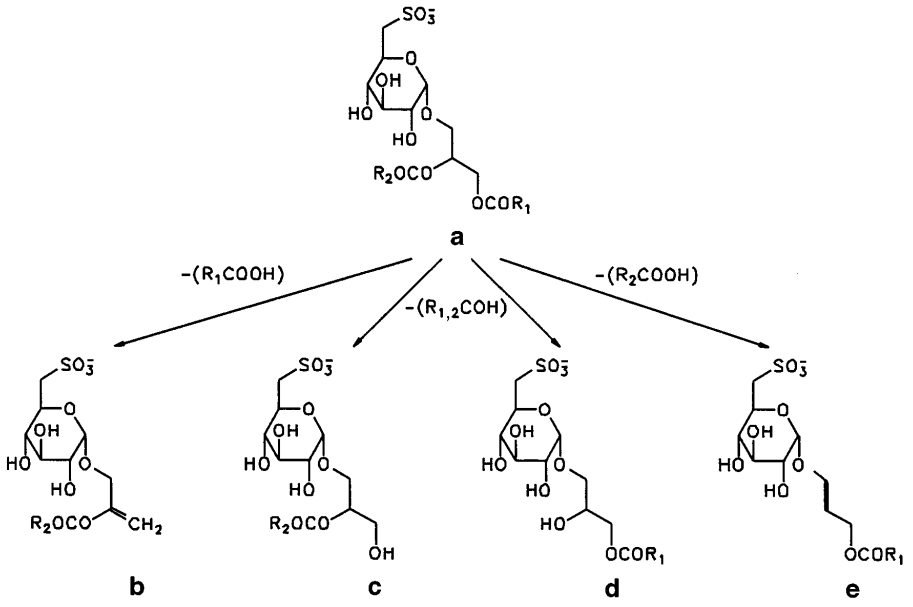


FIG. 5. Tandem mass spectrum of the $[M - H]^-$ ion of the major sulfoquinovosyl diacylglycerol (16:0/16:1 fatty acyl pairing) found in the *Heterosigma carterae* cell extract, taken from a combined chromatography/tandem mass spectrometry run.

Extensive interpretation of the LC/MS/MS data allowed complete assignment of the SQDG produced by *H. carterae* (Table 4). The positional assignments of the fatty acyl moieties listed in Table 4 were made on the basis of the relative intensities of the $[M - H - R_{sn\text{-}1}CO_2H]^-$ and $[M - H - R_{sn\text{-}2}CO_2H]^-$ fragment ions in the MS/MS spectrum of each SQDG. These assignments were supported by the positional assignments for the SQDG components with the 16:0/16:1 and 20:5/16:1 fatty acyl pairings as determined by interpretation of the NMR spectra and regioselective enzymatic hydrolysis (see below). It was found that, in general, the relative intensity of the fragment ion resulting from loss of the *sn*-1 fatty acyl moiety ($[M - H - R_{sn\text{-}1}CO_2H]^-$, *m/z* 535 in Figure 5) is significantly higher than the relative intensity of the fragment ion resulting from loss of the *sn*-2 fatty acyl group ($[M - H - R_{sn\text{-}2}CO_2H]^-$, *m/z* 537 in Figure 5). The only exception to this rule appears to be when a highly polyunsaturated fatty acyl group is attached to the *sn*-2 position. In this case, only a small difference in the relative intensity of the two fragment ions occurs.

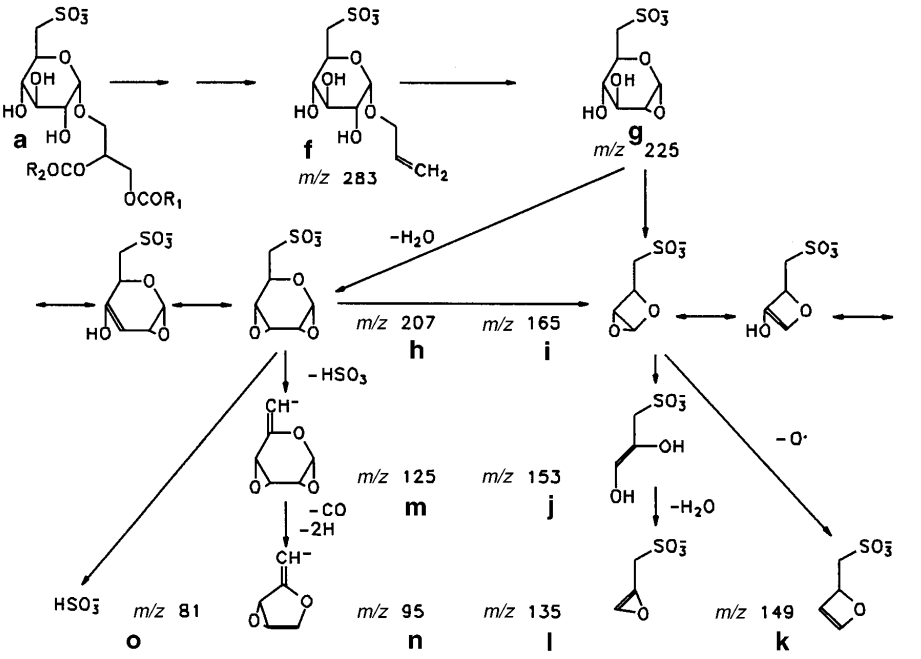
NMR analyses of SQDG fractions. To obtain SQDG components for structural analysis by NMR spectroscopy and for regioselective enzymatic cleavage of the *sn*-1 fatty acyl moiety, the SQDG mixture from *H. carterae* was separated by preparative LC. The fractions containing a mixture of three SQDG components having the 16:0/16:1 fatty acyl pairing (compounds **1**, **2**, and **3**), and the fraction containing mainly the SQDG with the 20:5/16:1 fatty acyl pair (substance **4**), were subjected to NMR analysis. The ^1H and ^{13}C NMR spectra of both fractions exhibited nearly identical chemical shifts for resonances assigned to the sulfoquinovosyl-glycerol moieties. The NMR assignments for the fraction containing the mixture of SQDG with the 16:0/16:1 fatty acyl pairing are listed in Tables 5 and 6, and were made on the basis of interpretation of one- and two-dimensional [(including ^1H-^1H correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), nuclear Overhauser enhancement spectroscopy, heteronuclear multiple quantum correlation (HMQC), and heteronuclear multiple bond correlation (HMBC)] NMR spectra. Where comparison was possible, the resonances observed for the sulfoquinovosyl-glycerol moieties are in excellent agreement with literature values (26).

The positional distribution of the fatty acyl groups in the mixture of SQDG having the 16:0/16:1 fatty acyl pairing was determined by two different methods, *viz.*, NMR spectroscopy and regioselective cleavage of the acyl group at the *sn*-1 position. By using 3:1 (vol/vol) CD_3OD/C_6D_6 as the solvent for NMR examination of the mixture of compounds **1**, **2**, and **3**, the resonance at 5.44 ppm, assigned to the proton at the *sn*-2 position of the glycerol moiety, could be resolved from the resonances attributed to the olefinic protons in the



SCHEME 1

16:1 acyl substituents. In the HMBC spectrum of the mixture, a correlation was observed between the proton resonance at 5.44 ppm and the carbon resonating at δ 174.96 ppm, thus allowing assignment of the latter resonance to the carbonyl carbon of the fatty acyl group attached to the *sn*-2 position. The methylene protons resonating at 2.32–2.35 ppm showed a correlation in the HMBC spectrum to the same carbonyl carbon (δ 174.96), thereby allowing assignment of these protons as the methylene protons on the carbon adjacent to the carbonyl carbon in the fatty acyl group attached at the *sn*-2 position. Similarly, correlations from the resonances at 4.28 ppm and at 2.26–2.27 ppm to the carbonyl carbon at 175.11 ppm proved the assignment of these resonances to the *sn*-1 position and its associated FA. The TOCSY spectrum of the mixture revealed that the protons resonating at 2.02 ppm (assigned to the allylic protons in the 16:1 fatty acyl groups; see Table 6) belonged to the same spin systems as those resonating at 2.32–2.35 ppm, a result which allowed assignment of



SCHEME 2

TABLE 5
Nuclear Magnetic Resonance Assignments of Substances 1–3

	Solvent					
	CD_3OD				CD_3OD/C_6D_6	
Positions	^1H	Sys.	*J* (Hz)	^{13}C	^1H	^{13}C
1	4.77	*d*	3.8	100.1	4.85	100.3
2	3.38	*dd*	3.8/9.8	73.5	3.53	73.6
3	3.62	*dd*	8.8/9.8	74.88	3.79	75.06
4	3.06	*dd*	8.8/9.9	74.90	3.22	75.13
5	4.05	*ddd*	2.1/9.2/9.9	69.8	4.21	69.9
6	2.90	*dd*	9.2/14.3	54.2	3.08	54.6
	3.37	*dd*	2.1/14.3		3.50	
1′	3.55	*dd*	6.4/10.8	67.1	3.64	67.4
	4.08	*dd*	5.3/10.8		4.19	
2′	5.30	*m*	na	71.7	5.44	71.8
3′	4.18	*dd*	6.9/12.0	64.3	4.28	64.5
	4.49	*dd*	3.0/12.0		4.59	
	FA attached to C_2' (*sn*-2)					
1				174.9		174.96
2	2.33	*m*	7.5/15.7	35.1	2.32	35.4
					2.35	
3	1.59	*m*	na	26.1	1.58	26.1
	FA attached to C_3' (*sn*–1)					
1				175.1		175.11
2	2.31	*m*	7.5/15.7	35.0	2.26	35.2
					2.27	
3	1.59	*m*	na[a]	26.1	1.58	26.1

[a]na, not available. For other abbreviations, see Table 4.

all three 16:1 fatty acyl groups exclusively to the *sn*-2 position.

Enzymatic cleavage of SQDG and analyses of the resulting sulfoquinovosyl monoacylglycerol (SQMG). To confirm this assignment, the 16:0 fatty acyl moiety at the *sn*-1 position of the mixture was regioselectively hydrolyzed using lipase from the fungus *Rhizopus arrhizus* (7,26). Negative ion liquid secondary ion mass spectrometry (LSIMS) analysis of the resulting SQMG gave an $[M - H]^-$ ion at *m/z* 553, clearly indicating that the remaining fatty acyl group was 16:1. However, NMR analysis of this SQMG (including ^1H-^1H COSY, HMBC, and HMQC spectra, see Table 7) revealed a mixture of compounds **5**, **6**, and **7** which have the remaining 16:1 fatty acyl moiety attached to the *sn*-1 position. This is clearly seen from the 4.16 ppm chemical shift of the methine proton attached to the *sn*-2 position, and the chemical shifts of 4.16 and 4.27 ppm observed for the methylene protons attached to the *sn*-1 position. The location of the 16:1 fatty acyl moiety

TABLE 6
Nuclear Magnetic Resonance Assignments of Substances 1–3 (solvent CD_3OD)

	16:0 (**1–3**)		16:1 n-3/Δ13 (in **3**)		16:1 n-5/Δ11 (in **2**)	
Position	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
4	1.3	30	1.3	30	1.3	30
5	1.3	30	1.3	30	1.3	30
6	1.3	30	1.3	30	1.3	30
7	1.3	30	1.3	30	1.3	30
8	1.3	30	1.3	30	1.3	30
9	1.3	30	1.3	30	1.3	30
10	1.3	30	1.3	30	2.02	28.2
11	1.3	30	1.3	30	5.33	130.6
					(*J* = 11.7 Hz)	
12	1.3	30	2.02	28.0	5.35	131.0
13	1.3	30	5.31	130.2	2.02	28.2
			(*J* = 10.6 Hz)			
14	1.29	33.1	5.34	132.4	1.28	30.1
15	1.29	23.8	2.04	21.5	1.27	24.0
16	0.88	14.5	0.93	14.8	0.89	14.2
	(*J* = 7.0 Hz)		(*J* = 7.6 Hz)		(*J* = 7.2 Hz)	

TABLE 7
Nuclear Magnetic Resonance Assignments of Substances 5–10

	5–7[a]		**8–10**[b]		**11**[a]	
Position	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	4.84	100.3	4.75	100.1	4.85	100.0
2	3.49	73.9	3.40	73.7	3.50	73.5
3	3.77	75.3	3.62	75.1	3.74	75.1
4	3.12	75.2	3.07	74.8	3.16	74.7
5	4.22	70.0	4.07	69.8	4.21	69.7
6	3.04	54.6	2.92	54.2	3.02	54.2
	3.47		3.33		3.44	
1′	3.47	70.0	3.52	67.3	3.44	70.5
	4.16		4.08		4.12	
2′	4.16	70.8	5.08	74.8	3.98	72.7
3′	4.16	66.6	3.72	62.2	3.68	64.0
	4.27					

[a]Solvent: 3:1 CD_3OD/C_6D_6.
[b]Solvent: CD_3OD.

at the *sn*-1 rather than the expected *sn*-2 position can be explained by an acyl migration favored by proton-donating solvents (28,29) since in this experiment, the SQMG had been purified using a C_{18} derivatized silica gel column followed by preparative LC, involving the use of triethylamine and water.

The enzymatic hydrolysis experiment was then repeated using an alternatve purification procedure in which, following hydrolysis, proteins and salts were precipitated by alcohols. The NMR spectra of the SQMG products were then run directly after filtration of the alcoholic solution. Using this alternative purification procedure, the product was determined to be an SQMG mixture composed of compounds **8**, **9**, and **10** (selected NMR assignments are listed in Table 7). In contrast to the NMR data for the SQMG containing **5**, **6** and **7**, the methine proton attached to the *sn*-2 position now resonated at 5.08 ppm and the signals assigned to the two *sn*-1 methylene protons had identical chemical shifts at 3.72 ppm. The isolation of either the *sn*-1 or *sn*-2 acylated product, depending on purification conditions, demonstrates the importance of conducting regioselective enzymatic cleavage and subsequent workup under carefully controlled conditions in order to obtain the expected *sn*-2 monoacylated product.

The *sn*-1 selective enzymatic cleavage followed by negative-ion LSIMS analysis was also applied to different SQDG fractions containing 14:0/16:1, 16:0/14:0, 18:3/16:1, and 20:5/16:0 FA pairings to yield an $[M - H]^-$ for the corresponding SQMG at *m/z* 553 (loss of 14:0 FA), *m/z* 527 (loss of 16:0 FA), *m/z* 553 (loss of 18:3 FA), and *m/z* 555 (loss of 20:5 FA), respectively. These results are in full accordance with the positional assignments obtained by the LC/MS/MS experiments described earlier (see Table 4).

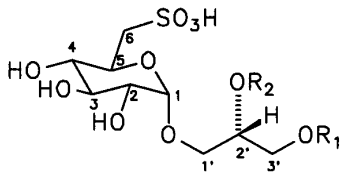
To confirm the structure of the sulfoglycoglycerol moiety, the mixture of **5**, **6,** and **7** was hydrolyzed under acid conditions to give the deacylated product **11**. The ^{1}H and ^{13}C NMR data for **11** (Table 7) are in excellent agreement with those reported for 3-*0*-(6-sulfo-α-D-quinovopyranosyl)-*sn*-glycerol (26,30). These data, together with comparison of the NMR data of the intact SQDG discussed earlier, support the relative stereochemistry of the sulfoglycoglycerol moiety as shown in Scheme 3. Although the data presented here do not exclude the possibility that the *H. carterae* SQDG sulfoglycoglycerol moiety actually has the opposite absolute configuration to that presented in structure **11**, it is unlikely that *H. carterae* would be the only organism to produce such a sulfoquinovosyl glycerol.

In conclusion, it has been shown that *H. carterae* contains numerous SQDG, all with the same sulfoquinovosyl-glycerol backbone substituted by various saturated, monounsaturated, and polyunsaturated FA. The most abundant SQDG contain the fatty acids 16:0, 16:1, and 20:5. Isolation of the individual SQDG was deemed impractical, so an LC/MS/MS system for their separation and identification was developed to elucidate their profile in extracts of *H. carterae*. The positional assignments of the fatty acyl moieties on the sulfoquinovosyl glycerol backbone could be made on the basis of the relative intensity of the $[M - H - R_{sn\text{-}1}CO_2H]^-$ and $[M - H - R_{sn\text{-}2}CO_2H]^-$ fragment ions in the LC/MS/MS spectra. The locations of carbon-carbon double bonds on the fatty acyl chains was determined from the GC/EI mass spectra of their nicotinyl derivatives. For monounsaturated fatty acyl chains, the configurations of these double bonds were determined by NMR analyses of the corresponding FAME. The combined results obtained from the different analytical techniques allowed an almost complete determination of the SQDG in *H. carterae.*

MATERIALS AND METHODS

All solvents were analytical grade, and purchased either from Baker (Phillipsburg, NJ), BDH (Toronto, Canada), or Sigma (St. Louis, MO). Distilled or Milli-Q-grade water was used for chromatography.

Extraction and isolation of SQDG. Hetersigma carterae (isolate 102R) was obtained from the Notheast Pacific Culture Collection, University of British Columbia, Vancouver, British Columbia, Canada. The cultures were grown in f/2



Substance no.	$\mathbf{R}_1$ (FA)[a]	$\mathbf{R}_2$ (FA)
1	16:0	16:1 Δ9 *cis*
2	16:0	16:1 Δ11 *cis*
3	16:0	16:1 Δ13 *cis*
4	20:5 Δ5, Δ8, Δ11, Δ14, Δ17 all-*cis*	16:1 Δ13 *cis*
5	16:1 Δ9 *cis*	H
6	16:1 Δ11 *cis*	H
7	16:1 Δ13 *cis*	H
8	H	16:1 Δ9 *cis*
9	H	16:1 Δ11 *cis*
10	H	16:1 Δ13 *cis*
11	H	H

[a]Abbreviation: FA, fatty acid.

SCHEME 3

seawater medium (31) at 22°C on a 16:8 h light/dark cycle under an irradiance of 80 mmol-photons $m^{-2}s^{-1}$ provided by 40-W cool-white fluorescent lamps. Wet cell material (160 g) was extracted into MeOH/$CHCl_3$ (7:3, vol/vol), yielding a total of 850 mg of SQDG after silica gel column chromatography, as described in Reference 7. A portion of this crude SQDG fraction was further fractionated on a Nova-Pak C_{18} LC column (7.8 mm i.d. × 300 mm; Waters Ltd., Milford, MA) using a linear gradient (7).

sn-*1 Regioselective enzymatic cleavage of the SQDG.* The enzymatic digestion is based on a procedure described in Reference 27 and is described in detail in Reference 7. Lipase type XI from *Rhizopus arrhizus* (Sigma) was used. Approximately 5 mg of each SQDG-fraction was incubated in Tris buffer (pH 7.4) at 37°C for 15 min. The reaction was stopped by adding isopropanol (15 mL), and the solvent was evaporated under reduced pressure. The residue was loaded onto a flash column (8 mm i.d.) packed with 2.7 g of C_{18} derivatized silica gel (Baker) and eluted with 20 mL water, 5 mL of a mixture of triethylamine, acetonitrile and water (1:1:8, to convert acidic compounds into their corresponding salts), 20 mL water, and finally 40 mL MeOH. The MeOH fraction was analyzed by mass spectrometry (LSIMS). For NMR experiments, this fraction was further purified by LC using a Nova-Pak C_{18} column (4.5 × 300 mm) with an isocratic solvent system containing MeOH/water (9:1), a Waters 510 pump combined with a Waters refractive index detector RI 410, and a flow rate of 1.2 mL/min. In this way 1 mg of SQMG was obtained (R_t 3.8 min), liberated FA eluted at R_t 12.8 min and a small amount of undigested SQDG eluted at R_t 20.9 min. Note that this procedure resulted in a migration of the *sn*-2 fatty acyl moiety onto the *sn*-1 position after enzymatic digestion.

The alternative procedure, adopted to yield **8**–**10**, was to stop the enzymatic reaction with isopropanol (20 mL), then evaporate the solvent under reduced pressure. The residue was dissolved in 2 mL MeOH and transferred to a clean vial, and the solvent evaporated by a stream of dry nitrogen. The residue was dissolved in 1 mL MeOH and filtered (0.5 μm filter cartridge). NMR analysis of the protein-free filtrate was then performed on the same day.

Formation of FAME. Twenty mg of the purified SQDG fraction was dissolved in 2 mL 0.5 M NaOH/MeOH and equilibrated for 30 min at room temperature. A 2-mL aliquot of BF_3 in MeOH (20%) was added, and after 30 min the volume was reduced to 0.5 mL by a stream of nitrogen. Saturated aqueous NaCl solution (20 mL) was added and the mixture extracted three times with 20 mL *n*-hexane each. The hexane layers were combined and volume reduced to 25 mL under reduced pressure. The hexane fraction was washed with 20 mL H_2O, dried over Na_2SO_4 for about 10 min, and solvent was evaporated under reduced pressure to yield 2.6 mg of a colorless oil which was analyzed by GC/MS.

MS. LSIMS and GC/MS experiments were performed using a VG ZAB-EQ mass spectrometer (Micromass Ltd., Manchester, United Kingdom) coupled to a Hewlett-Packard Series II gas chromatograph with on-column injector (Palo Alto, CA). For LSIMS experiments, a 20 keV beam of Cs^+ ions and a triethanolamine matrix were used. MS/MS experiments were performed in the first field-free region by a linked scan at constant B/E. Ar was used as collision gas at a pressure which attenuated the ion beam by 50% and at a collision energy of 8 keV. EI experiments were performed at an ion source temperature of 200°C and an ionizing electron energy of 70 eV. For further details see Reference 7.

LC/MS/MS experiments were performed using a Hewlett-Packard 1090 HPLC system combined with a PE-SCIEX API III+ triple quadrupole mass spectrometer (Concord, ON,

Canada) operated in negative ion ionspray mode. A Zorbax SB C_{18} LC column (0.21 × 15 cm) was used at a flow rate of 0.2 mL min^{-1}. The optimal LC separation was achieved using 0.03% NH_4OH in H_2O and CH_3CN as solvents in linear gradients such that their respective percentages were 70:30 initially; 50:50 at 10 min; 20:80 at 30 min; 0:100 at 35 min; and 70:30 at 45 min.

NMR spectroscopy. NMR spectra at 20°C were recorded on a Bruker AMX-500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.13 MHz (^1H), 125.7 MHz (^{13}C), using 5-mm sample tubes (Wilmad Glass, Buena, NJ; model 535pp) and standard Bruker pulse sequences. Solvents CD_3OD or 3:1 CD_3OD/C_6D_6 were used as noted in the text or tables. Chemical shifts were referred to CHD_2OD at 3.30 ppm (^1H), or $^{13}CD_3OD$ at 49.0 ppm (^{13}C).

Acquisition conditions, one-dimensional spectra: (^1H) spectral width (SW) 4098 Hz, acquisition time (AQ) 4.0 s, 66° pulse, delay after acquisition (D1) 2 s, decoupling field where used *ca.* 250 Hz; (^{13}C) SW 33,333 Hz, AQ 0.98 s, 40° pulses, Waltz decoupling of ^1H, D1 0.1 s. Spectra were processed with zero filling after exponential weighting (line broadening 0.2 Hz for ^1H, 0.5 Hz for ^{13}C) or resolution enhancement by Lorentz-Gauss multiplication. Couplings of some protons were obtained by simulation.

Two-dimensional spectra (^1H TOCSY and double-quantum filtered COSY): SW 3676 Hz, AQ 0.07 s, D1 2.0 s, 512 × 512 increments, TOCSY spin lock for 160 ms at field strength 8 kHz, processed with zero filling and apodization by 90° shifted sine-bell squared in both dimensions; ($^1H/^{13}C$ HMQC and HMBC) ^1H SW 3676 Hz, ^{13}C SW 16,600 or 22,726 Hz, AQ 0.07 s, D1 1.5 s, 512 × 1024 increments, zero filling both dimensions, apodization as above for HMQC, unshifted sine-bell squared in both dimensions for HMBC. Delay for HMBC 60 ms and 90 ms.

Isolated compounds. 1′-*O*-(6-desoxy-6-sulfo-α-D-glucopyranosyl)-2′-*O*-(hexadeca-9- enoyl)-3′-*O*-hexadecanoyl-*sn*-glycerol, **1**; 1′-*O*-(6-desoxy-6-sulfo-α-D-glucopyranosyl)-2′-*O*-(hexadeca-11-enoyl)-3′-*O*-hexadecanoyl-*sn*-glycerol, **2**; 1′-*O*-(6-desoxy-6-sulfo-α-D-glucopyranosyl)-2′-*O*-(hexadeca-13-enoyl)-3′-*O*-hexadecanoyl-*sn*-glycerol, **3**; total yield 100 mg; NMR data, see Tables 5 and 6; MS see Table 4 and References 7, 25.

1′-*O*-(6-desoxy-6-sulfo-α-D-glucopyranosyl)-2′-*O*-(hexadeca-13-enoyl)-3′-*O*-(eicosapenta-5,8,11,14,17-enoyl)-*sn*-glycerol, **4**; 69.2 mg. ^1H NMR data of the eicosapentaenoic acid moiety: δ 2.25 (2H, *t*; H at C_2), δ 1.60 (2H, *m*; H at C_3), δ 2.02 (4H, *m*; H at C_4, C_{19}), δ 5.36 (10H, *m*; olefinic protons), δ 2.80 (8H, *m*; H at C_7, C_{10}, C_{13}, C_{16}), δ 0.96 (3H, *t*, J = 7.6 Hz; H at C_{20}). All other shifts are identical with those given in Table 5. The signal at δ 4.49 (^1H at C_3') is slightly downfield-shifted (δ 4.50). ^{13}C NMR data: methyl groups: δ 15.0, δ 15.1; methylene groups: δ 21.7–δ 35.4; olefinic carbons: δ 128.3–δ 133.0 ($CDCl_3/C_6D_6$, 3:1); all other values are identical with those given in Table 5. MS data: see Table 4.

1′-*O*-(6-desoxy-6-sulfo-α-D-glucopyranosyl)-3′-*O*-hexadecaenoyl-*sn*-glycerols, **5**–**7**; yield after high-performance liquid chromatography separation *ca.* 1 mg. Enzymatic cleavage and purification are described in Reference 7. NMR data: see Table 7. MS data are given in Reference 7.

1′-*O*-(6-desoxy-6-sulfo-α-D-glucopyranosyl)-2′-*O*-hexadecaenoyl-*sn*-glycerol, **8**–**10**; yield *ca.* 5 mg. NMR data, see Table 7; $[M - H]^-$ at *m/z* 553.4; fragmentation series identical with **4**–**7**.

1′-*O*-(6-desoxy-6-sulfo-α-D-glucopyranosyl)-*sn*-glycerol, **11**; yield 3.6 mg. Acid-catalyzed hydrolysis: 15 mg of the SQDG (16:1/16:0) were dissolved in 5 mL absolute MeOH and a few drops of trifluoroacetic acid were added. Compound **11** was purified by LC as described in Reference 7. NMR data are given in Table 7; further analytical data: see References 26 and 30.

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Measurement of Apolipoprotein B in Various Cell Lines: Correlation Between Intracellular Levels and Rates of Secretion

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ABSTRACT: We have standardized simple but sensitive enzyme-linked immunoassays to understand a relationship between intracellular levels and secretion rates of apoB. The assays were based on commercially available antibodies and were specific to human apoB. A monoclonal antibody, 1D1, was immobilized on microtiter wells and incubated with different amounts of low density lipoproteins to obtain a standard curve. Conditioned media were added to other wells in parallel, and the amount of apoB was quantitated from a linear regression curve. To standardize conditions for the measurement of intracellular apoB, cells were homogenized and solubilized with different concentrations of taurocholate. We found that 0.5% taurocholate was sufficient to solubilize all the apoB in HepG2, Caco-2, and McA-RH7777 cells. Next, a standard curve was prepared in the presence of taurocholate and used to determine intracellular levels of apoB in different cell lines. The intracellular levels (pmol/mg cell protein) and the rates of secretion (pmol/mg/h) of apoB100 were positively correlated (r^2= 0.81, P = 0.0009) in HepG2 cells. Furthermore, a positive correlation (r^2= 0.88, $P < 0.0001$) was found between intracellular and secreted apoB42 in stably transfected McA-RH7777 cells. In contrast, no correlation was observed for human apoB28 and apoB18 in stably transfected cells that were secreted either partially associated or completely unassociated with lipoproteins. These studies indicated that the rate of secretion of lipid-associated apoB, but not the lipid-free apoB, was tightly controlled.
Lipids 32, 1113–1118 (1997).

Apolipoprotein B (apoB) is an essential protein constituent for the assembly of triglyceride-rich lipoproteins by the liver and intestine. The size and flotation properties of lipoproteins have been related to the length of apoB. The apoB polypeptides smaller than 23% are secreted as lipid-poor proteins (1,2). As the length of the apoB polypeptide increases, the size of lipoprotein particles increases but the flotation density decreases (1–5). The apoB mRNA levels do not change by various dietary and metabolic conditions that affect plasma apoB levels. Plasma apoB levels are altered by affecting the translational and posttranslational events during lipoprotein biosynthesis. For example, apoB100 synthesis is decreased in streptozotocin-treated diabetic rats (6), insulin-treated rats and HepG2 cells (7,8), cyclosporin-treated HepG2 cells (9), and in cells transfected with human recombinant apoB48 cDNA (10). Different lipids modulate intracellular degradation and secretion of apoB [for reviews, (11–15)]. Fatty acids modulate the secretion of apoB100 in hepatoma cell lines by altering the rates of intracellular degradation (16–18). To determine a relationship between intracellular levels of apoB and rates of secretion of apoB-containing lipoproteins, we standardized methods to measure intracellular and secreted apoB.

MATERIALS AND METHODS

Materials. Cell culture media were from Bio-Whittaker (Walkersville, MD). Fetal bovine (FBS) and horse serums were obtained from BioFluids (Rockville, MD), and Life Technologies, Inc. (Gaithersburg, MD), respectively. A monoclonal antibody, 1D1, was purchased from Dr. Yves Marcel of the Ottawa Heart Institute, Canada. Polyclonal sheep antihuman apolipoprotein (apoB) (#726494) antibodies were obtained from Boehringer Mannheim (Indianapolis, IN). Alkaline phosphatase-labeled antisheep IgG (#56991) was from Cappel (Durham, NC). Neomycin analog G418 was from Gibco/BRL (Gaithersburg, MD). Taurocholic acid (#T4009) was from Sigma Chemical Co. (St. Louis, MO). Microtiter plates (#1424586) were from Dynatech Laboratories Inc. (Alexandria, VA).

Low density lipoproteins (LDL). LDL (d = 1.02–1.06 g/L), prepared from fresh human plasma by sequential ultracentrifugation, were obtained from the core laboratory of the Department of Biochemistry and analyzed by agarose gel electrophoresis (Beckman, Brea, CA), gel filtration chromatography, and amino acid analysis. To study the gel elution profile, it was applied on a Superose-6 HR 10/30 column (Pharmacia, Uppsala, Sweden) at a flow rate of 0.25 mL/min. The elu-

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Abbreviations: Apo, apolipoprotein; BSA, bovine serum albumin; ELISA, enzyme-linked immunoassay; FBS, fetal bovine serum; LDL, low density lipoproteins; MEM, minimum essential medium; PBS, phosphate buffered saline; PBS-Tween, PBS containing 0.05% Tween-20.

tion of proteins was continuously monitored by measuring absorbance at 280 nm. LDL were further analyzed by amino acid analysis by the Wistar Protein Microchemistry Facility, Wistar Institute (Philadelphia, PA). For this purpose, 2 μg of LDL were hydrolyzed with 6 N HCl, 1% phenol for 1 h at 160°C, followed by manual phenylthiocarbamyl derivatization and high-performance liquid chromatography separation as described by Ebert (19).

Cells. HepG2, Caco-2 and McA-RH7777 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in minimal essential medium (MEM) containing 10% FBS, MEM containing 20% FBS, and Dulbecco's modified MEM containing 10% FBS and 10% horse serum, respectively. McA-RH7777 cells (1,10,20) stably transfected with vectors expressing different truncated human apoB cDNA were kindly provided by Dr. Zemin Yao of the Ottawa Heart Institute, Canada. These cells were cultured in Dulbecco's modified MEM containing 10% FBS, 10% horse serum, and 200 μg/mL of G418. All media contained 1% antibiotic-antimycotic cocktail (Gibco/BRL).

Quantification of secreted and intracellular human apoB by enzyme-linked immunoassay (ELISA). Microtiter wells were coated (2 h, room temperatures) with a monoclonal antibody, 1D1, [100 μl, 1:1000 dilution in phosphate buffered saline (PBS), pH 7.4], washed (3×) with PBS containing 0.05% Tween 20 (PBS-Tween). Conditioned media or various concentrations of human LDL in PBS-Tween were added to each well in triplicate and incubated for 2 h. The wells were washed (3×) with PBS-Tween, incubated (1 h) with sheep antihuman apoB polyclonal antibodies (1:2000 dilution in PBS-Tween), washed (3×) with PBS-Tween, and incubated with purified rabbit anti-sheep IgG labeled with alkaline phosphatase (1:1000 dilution, 1 h). The anti-sheep IgG increased the sensitivity of detection. Finally, the wells were washed twice with PBS-Tween, PBS, and 10 mM ethanolamine, 0.5 mM $MgCl_2$, pH 9.5, and incubated with 100 μL of *p*-nitrophenyl phosphate (1 mg/mL in 10 mM ethanolamine, 0.5 mM $MgCl_2$, pH 9.5) for various times. The absorbance at 405 nm was determined using a Microplate Reader (Dynatech Labs, Chantilly, VA). To determine intracellular apoB levels, cells were washed with PBS, scraped, pelleted by centrifugation, resuspended in PBS, and homogenized (30–60 s, setting 3, 4°C). An aliquot of the cell homogenate was saved for protein determinations, and the rest was solubilized with 0.5% (wt/vol) taurocholate in PBS by rotating samples overnight at 4°C. The solubilized mixture was centrifuged (12000 rpm, 4°C, 5 min), and supernatant was used for apoB determinations. Standard curves were prepared using indicated amounts of taurocholate.

Other measurements. Proteins were determined using the Coomassie Plus reagent (Pierce Chemical Co., Rockford, IL) with bovine serum albumin (BSA) as a standard. The statistical and linear regression analyses were performed using Prism2 (Graphpad, San Diego, CA).

RESULTS AND DISCUSSION

Characterization and measurement of human LDL. Human plasma LDL were used as a standard in developing ELISA assays for apoB. Lipoproteins were characterized to ensure their purity. The purified LDL migrated as a single band during agarose gel electrophoresis and eluted as a single peak during Superose-6 column chromatography (data not shown). The expected and observed amino acid compositions were similar (data not shown), indicating that the purified LDL mainly contained apoB100. Next, we evaluated the use of Coomassie Plus reagent in the measurement of apoB since amino acid analysis is not expected to be practical in every laboratory. The LDL protein measured by amino acid analysis was higher by 12.5% in two different determinations compared to that obtained by Coomassie Plus reagent. These studies suggested that Coomassie Plus reagent can be used to quantify apoB.

Quantitation of secreted apoB. The sandwich ELISA technique developed (10) to quantify secreted apoB was based on a monoclonal antibody, 1D1, that recognizes an epitope in the *N*-terminus (amino acids 474–539) of human apoB (21,22). The monoclonal antibody recognizes all subclasses of very low density lipoprotein and LDL, and delipidated apoB with similar association constant of 10^{-9} M (23–25). Because of these properties, it has been used to detect various apoB polypeptides (1,10) and was selected for the development of an ELISA. We observed that the detection of LDL was linear up to 100 ng and reached a saturation at 340 ng of LDL (data not shown). Routinely, 1 to 10 ng of LDL were used to generate standard curves. The standard curves were subjected to linear regression analyses; the r^2 value in various experiments ranged between 0.92 and 0.99. Sensitivity of the assays could be increased by using 200 μL of media instead of the usual 100 μL during all steps, and using lower amounts of human LDL (0.5–5 ng of protein/well) for standard curves coupled with increased time for color development. The assay is specific to human apoB because 1D1 is specific to human apoB (21,22) and does not recognize rat, bovine, or horse apoB. Furthermore, human apoB determinations were not affected by the presence of FBS, horse serum, or BSA in the medium. This procedure has been used to measure apoB levels in plasma and various lipoprotein fractions (data not shown). The method described here is more sensitive, does not require affinity purification of antibodies, and is rapid compared to a recently described ELISA method for apoB detection (26). Hahn *et al.* (26) evaluated the use of monoclonal antibodies as capture antibodies and found that they were less effective than affinity-purified polyclonal antibodies. A possible reason for the poor efficiency may be that the monoclonal antibodies used in their studies recognized only lipid-associated apoB, and the recognition was modulated by the amounts of lipids present in lipoprotein particles (23,25). In our studies, we selected a monoclonal antibody, 1D1, that recognizes lipid-free and lipid-associated apoB with equal affinity (23–25).

Measurement of intracellular apoB. To develop a method for the quantitation of intracellular apoB, we evaluated different detergents for their use in ELISA. Deoxycholate (1%) and sodium dodecyl sulfate (1%) inhibited the detection of apoB by ELISA, whereas Triton X-100 (1%) and taurocholate had no significant effect (data not shown). We concentrated on

sodium taurocholate because it had been used for the measurement of microsomal apoB (27). Furthermore it has a high critical micellar concentration, does not induce protein denaturation, and has been used for the solubilization of membrane enzymes (28). First, we determined the feasibility of using taurocholate for measuring apoB in LDL. For this purpose, ELISA was performed in the presence of different concentrations of taurocholate (data not shown). At low concentrations (0.25%, wt/vol), taurocholate did not affect the slope of the curve compared to control (Table 1). However, at higher concentrations, taurocholate decreased the slope, suggesting the requirement of a standard curve for each taurocholate concentration. What is more important, the linearity of the assay was not affected by the presence of taurocholate. The *P* values after a two-tail test were <0.0001. Thus, apoB could be reliably measured in the presence of taurocholate.

Second, conditions were optimized to find out the maximum amount of taurocholate required for the detection of intracellular apoB in HepG2 and differentiated Caco-2 cells (Fig. 1). No apoB could be detected in cellular homogenate in the absence of taurocholate, indicating that the majority of the intracellular apoB was not accessible to antibodies under these conditions. Increasing concentrations of taurocholate resulted in increased detection of intracellular apoB. For HepG2 cells, 0.1% taurocholate was sufficient for the maximum solubilization of intracellular apoB (Fig. 1A), whereas for differentiated Caco-2 cells 0.3 to 0.4% taurocholate yielded maximum detection (Fig. 1B). In subsequent experiments, 0.5% taurocholate was used to ensure complete solubilization.

Third, we determined the feasibility of using taurocholate to quantify human apoB in McA-RH7777 cells stably transfected with various truncated forms of apoB (1,10). To determine the effect of taurocholate on the measurement of human apoB in McA-RH7777 cell homogenate, known amounts of LDL were added to washed cell monolayers of nontransfected cells. Cells were homogenized, solubilized, and used to detect human apoB. The recovery of added apoB using 0.5% taurocholate was 87%. These studies indicated that taurocholate could be used to solubilize intracellular or added apoB in various cells and to quantitate it by ELISA.

TABLE 1
Statistical Parameters for the Standard Curves Generated to Study the Effect of Different Concentrations of Taurocholate on Apolipoprotein B (apoB) Determinations[a]

Taurocholate (wt/vol)	Slope	Y intercept	r^2	Sy.x
0%	0.063 ± 0.003	0.080 ± 0.026	0.987	0.038
0.25%	0.066 ± 0.002	0.016 ± 0.018	0.994	0.027
0.50%	0.048 ± 0.002	0.027 ± 0.017	0.990	0.025
0.75%	0.053 ± 0.001	0.006 ± 0.005	0.999	0.008

[a]Microtiter wells were coated in triplicate with monoclonal antibody, 1D1 (purchased from Dr. Yves Marcel of the Ottawa Heart Institute, Canada), and incubated with different amounts of low density lipoprotein in the presence or absence of various concentrations of taurocholate (Sigma Chemical Co., St. Louis, MO). Subsequent steps for the determination of apoB (Boehringer Mannheim, Indianapolis, IN) were as described in the Materials and Methods section. The lines were subjected to linear regression analyses. Statistical parameters of the curves are summarized. r^2, coefficient of determinations; Sy.x, standard deviation of residuals from the line.

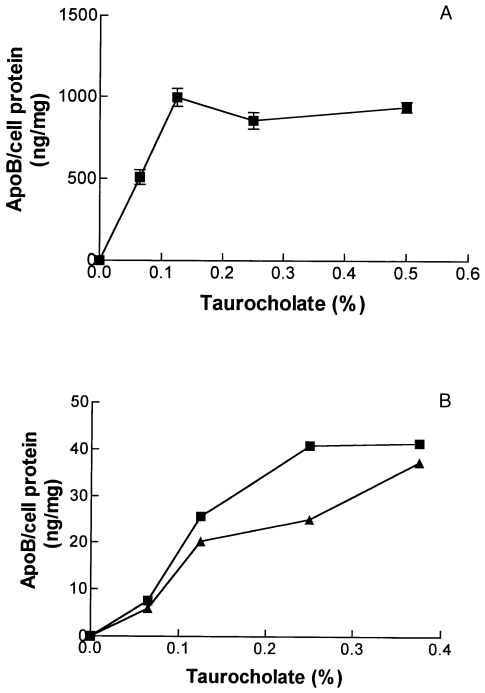

FIG. 1. Effect of different concentrations of taurocholate on the solubilization of intracellular apolipoprotein B (apoB). (A) HepG2 cells were plated in three flasks and used after they reached confluency. Means ± standard deviations of triplicate determinations are presented. (B) Caco-2 cells were plated in two flasks and allowed to differentiate for 21 d. These cells secreted 0.02–0.06 pmol of apoB/mg/h. Cells from these flasks were used to determine intracellular apoB levels. The data from both flasks are presented. To determine intracellular apoB, cells were scraped in phosphate buffered saline, homogenized, and distributed in different aliquots. The aliquots were incubated overnight at 4°C with indicated concentrations of taurocholate, centrifuged (12000 rpm, 5 min, 4°C), and the supernatant was used for enzyme-linked immunoassay. Different standard curves were prepared with indicated concentrations of taurocholate, and intracellular apoB levels were determined.

Intracellular levels and secretion rates of apoB polypeptides in different cell lines. We then used these techniques to quantitate intracellular levels and secretion rates of apoB in HepG2 cells at steady state (Table 2). The intracellular levels (0.43 ± 0.08 pmol apoB/mg cell proteins) of apoB found in the present study were similar to those reported by Hahn *et al.* (26) but were significantly higher than those reported by Byrne *et al.* (29). The lower values in the later studies may be due to the use of a detergent that might not have solubilized all the intracellular apoB. In contrast, the secretion rates (0.11 ± 0.01 pmol/mg/h) observed in the present study are similar to those of Byrne *et al.* (29) but significantly different from those of Hahn *et al.* (26). In the later studies, secretion rates were studied in the first 6 h after a media change and thus may not represent steady-state rates. The higher rates observed in that study also suggest that media changes may stimulate secretion of apoB; however, no attempts were made to study this phenomenon. In addition, we found that the apoB secreted per hour was a small fraction (0.2) of the intracellular levels.

Subsequently, attempts were made to quantitate C-terminally truncated apoB polypeptides. Since we used human

TABLE 2
The Steady-State Intracellular Levels and Secretion Rates of Different apoB Polypeptides in Various Cell Lines[a]

Cell lines–length of apoB polypeptides[f]	n[b]	Molecular weight (10^{-3} Da)	Cell protein (mg)	Intracellular[c] (pmol/mg)	Secretion rate[d] (pmol/mg/h)	Fraction secreted[e] (h)
HepG2-B100[g]	9	512	1.11 ± 0.32	0.43 ± 0.08	0.11 ± 0.01	0.20
McA-RH7777-B42[h]	12	215	4.01 ± 0.40	0.44 ± 0.06	0.20 ± 0.03	0.31
McA-RH7777-B28[h]	8	143	3.48 ± 0.86	0.55 ± 0.08	0.36 ± 0.07	0.39
McA-RH7777-B18[h]	8	92	2.60 ± 0.21	4.54 ± 0.50	2.28 ± 0.63	0.33

[a]For each experiment, cells were plated in 25-cm^2 flasks and incubated for various times (24–60 h). ApoB was determined in the medium and cells by enzyme-linked immunoassay as described in the Materials and Methods section.
[b]Number of flasks used in different experiments.
[c]Intracellular apoB was measured after solubilization of cell monolayers with 0.5% taurocholate as described in the Materials and Methods section.
[d]The secreted apoB was measured as described in the Materials and Methods section.
[e]Secretion rate/(intracellular + secretion rate).
[f]Various cell lines and human apoB polypeptides secreted by these cells are indicated. See Table 1 for abbreviation and other company suppliers.
[g]American Type Culture Collection (Rockville, MD).
[h]Kindly provided by Dr. Zemin Yao (Ottawa Heart Institute, Canada).

LDL as a standard, the number of molecules calculated for truncated apoB polypeptides was probably an underestimation. In the present assays, the capture of the apoB polypeptides by monoclonal antibodies, 1D1, was on molar basis, but the detection using polyclonal antibodies was not. The epitopes for the polyclonal antibodies are expected to be present throughout the apoB100, and they are expected to be absent to various degrees in truncated polypeptides. Thus, values for intracellular and secreted amounts for the same polypeptide are comparable, but quantitative comparisons between different polypeptides are not valid.

Next, we studied the rate of secretion of different C-terminally truncated apoB polypeptides in stably transfected McA-RH7777 cells (Table 2). In contrast to Hep G2 cells, the fraction of apoB polypeptides secreted by McA-RH7777 cells was higher (0.31 to 0.39), indicating that McA-RH7777 cells secreted shorter peptides more efficiently than the secretion of apoB100 by HepG2 cells. This probably was not due to more efficient secretion of shorter peptides because Parhofer *et al.* (30) have observed that the secretion rates of truncated polypeptides decrease with decrease in length. The most likely explanation is that these two cell lines process apoB polypeptides differently during intracellular transport.

Correlations between intracellular steady-state levels of apoB and rate of secretion of lipid-associated apoB. Until now significant information concerning the role of intracellular degradation and secretion of apoB from different cell lines has been obtained using metabolic labeling involving pulse-chase analysis (1,16,17,31). However, the relationship between intracellular apoB levels and rates of secretion is not known. We observed a strong correlation between intracellular and secreted apoB100 in HepG2 cells with an r^2 value of 0.81, and the slope of the curve was significantly ($P = 0.0009$) different from zero (Fig. 2A). Similar observations have been

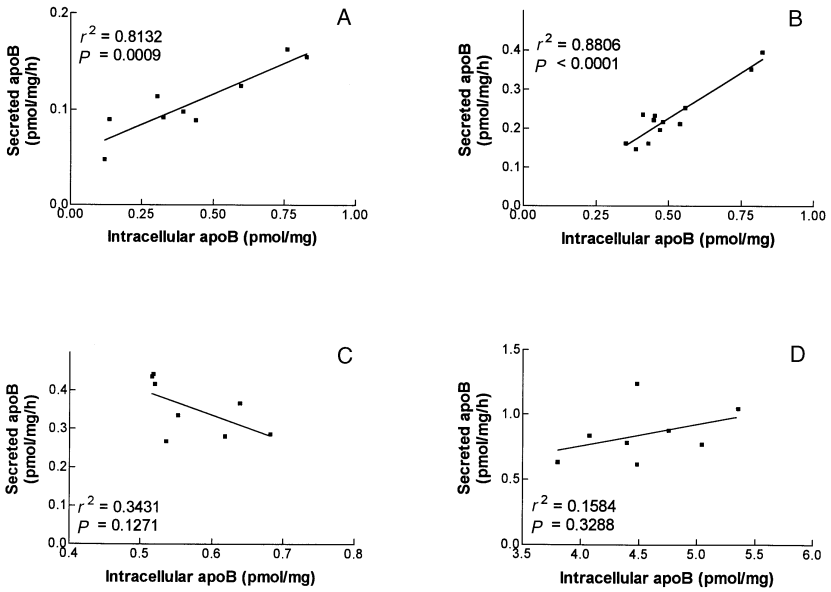

FIG. 2. Correlation between intracellular levels and secretion rates of apoB in cultured cells. Cells were plated in different flasks. At different times, apoB was measured in cells and media. The intracellular levels of apoB were plotted against secreted apoB and subjected to linear regression analysis. (A) HepG2 cells and media were obtained from nine different flasks. McA-RH7777 cells stably transfected with human recombinant (B) apoB 42, (C) apoB 28, and (D) apoB18 were plated in 12, 8, and 8 flasks, respectively. Flasks were harvested at different times of incubation and the amount of human apoB was determined in cells and media. See Figure 1 for abbreviation.

made before (29). Next, we studied the relationship between the lengths of apoB and their secretion rates. The secretion of apoB42, which is secreted as a lipoprotein particle, was significantly correlated ($r^2 = 0.88$) with intracellular levels (Fig. 2B). In contrast, no significant correlation between intracellular levels and secretion rates was observed for apoB28 and apoB18 (Fig. 2C and 2D); these polypeptides are secreted partially or completely in lipid-free forms (1–3). These data suggest that the rate of secretion of lipidated apoB may be more tightly controlled than apoB polypeptides secreted unassociated with lipids. It is tempting to speculate that cells may sense their environment and control the biosynthesis of apoB-containing lipoproteins.

In summary, we have standardized conditions to quantify secreted and intracellular apoB100 and its various truncated forms. We observed that the rates of secretion of lipidated apoB polypeptides are positively correlated with their intracellular levels. It remains to be determined whether such a correlation exists for other apolipoproteins secreted associated with lipids. Furthermore, mechanisms involved in determining the correlations should be explored.

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Oxidation Reactions of Acetylenic Fatty Esters with Selenium Dioxide/*tert*-Butyl Hydroperoxide

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ABSTRACT: Reaction of methyl undec-10-ynoate (**1**) with selenium dioxide/*tert*-butyl hydroperoxide (TBHP) in aqueous dioxane gave methyl 9-oxo-undec-10-ynoate (**2**, 9%) and 9-hydroxy-undec-10-ynoate (**3**, 60%), while methyl octadec-9-ynoate (**4**) yielded mixtures of positional isomers of mono-keto (*viz.* methyl 8-oxo- and 11-oxo-octadec-9-ynoate, **5**, 5%), hydroxy-keto (*viz.* methyl 8-hydroxy-11-oxo- and 11-hydroxy-8-oxo-octadec-9-ynoate, **6**, 10%), and dihydroxy (*viz.* methyl 8,11-dihydroxy-octadec-9-ynoate, **7**, 24%) derivatives. Similar treatment of a conjugated diacetylenic fatty ester (methyl octadeca-6,8-diynoate, **8**) furnished a mixture of methyl 5-oxo- and 10-oxo-octadeca-6,8-diynoate (**9**, 12%) and a complex mixture of very polar products. Reaction of methyl octadec-11*E*-en-9-ynoate (methyl santalbate) (**10**) with selenium dioxide/TBHP in aqueous dioxane gave exclusively a mixture of regiospecific products, *viz.* methyl 8-oxo-octadec-11(*E*)*Z*-en-9-ynoate (**11**, 6%) and methyl 8-hydroxy-octadec-11*E*-en-9-ynoate (**12**, 70%). The structures of the various products were determined by a combination of spectroscopic and mass spectral analyses.

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Selenium dioxide is commonly used to oxidize the α-methylene carbon atoms adjacent to a double bond to form allylic hydroxy derivatives (1). One serious complication of this reaction is the difficulty of removing the colloidal reduced forms of selenium after the reaction. To circumvent this technical obstacle, an improved hydroxylation method has been developed by Umbreit and Sharpless (2) where *tert*-butyl hydroperoxide (TBHP) is used as a re-oxidant in combination with selenium dioxide. This modified procedure has been successfully applied to the allylic hydroxylation of many olefinic compounds including unsaturated olefinic fatty esters (3–6). For example, in the reaction of methyl oleate with selenium dioxide and TBHP, a mixture of methyl 8- and 11-hydroxy-octadec-9*Z*-enoate and methyl 8,11-dihydroxy-octadec-9*Z*-enoate is obtained (4). In such hydroxylation reactions, allylseleninic acid has been identified as an intermediate species (7–9). Aldehydes, ketones, and carboxylic esters also can be subjected to α-oxidation by selenium dioxide (10). Allylic rearrangement reactions often are found to accompany this type of reaction, and *cis* double bonds tend to isomerize to the *trans* configuration (1,2). While allylic hydroxylation reactions involving olefinic compounds have been extensively documented in the chemical literature, comparatively little is reported on the oxidation of acetylenic fatty acids by selenium dioxide/TBHP. Chabaud and Sharpless (11) have shown that oxidation of acetylenes by this procedure yields α,α´-dioxygenated derivatives.

Our continued interest in the oxidation of unsaturated fatty acids has led us to study the chemical effects of selenium dioxide/TBHP on unsaturated fatty acid substrates containing either a terminal acetylenic, an internal acetylenic, a conjugated diacetylenic, or a conjugated olefinic-acetylenic (enyne) system.

MATERIALS AND METHODS

Thin-layer chromatography was performed on microscope glass plates coated with silica gel G (*ca.* 0.1-mm thick) and a mixture of *n*-hexane/diethyl ether (4:1, vol/vol) as the developer. Components on the microplates were viewed by exposing them to iodine vapor. Column chromatographic separation was accomplished using silica gel (Merck Art. 7730, type 60, 70–230 mesh; Darmstadt, Germany) as the adsorbent using gradient elution with mixtures of *n*-hexane/diethyl ether as the eluent. Infrared (IR) spectra were measured on a Shimadzu model IR-470 spectrophotometer (Shimadzu Corp., Kyoto, Japan) on neat samples placed between NaCl discs. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DPX_{300} (300 MHz) Fourier transform NMR spectrometer (Bruker, Fallanden, Switzerland) from solutions in deuteriochloroform (CDCl3) (0.2–0.3 mM) with tetramethylsilane (TMS) as the internal reference standard. Chemical shifts are given in δ-values in ppm downfield from TMS (δ_{TMS} = 0 ppm). Low- and high-resolution mass spectra were recorded on a Finnigan Mat 95 mass spectrometer (Finnigan Mat Corp., San Jose, CA). The following acetylenic fatty esters were prepared by methods described elsewhere: methyl undec-10-ynoate (**1**) (12), methyl octadec-9-ynoate (**4**) (13), and methyl octadeca-6,8-diynoate (**8**) (14). Methyl octadec-11*E*-en-9-ynoate (**10**) was isolated from *San-*

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Abbreviations: ^{13}C NMR, carbon-13 nuclear magnetic resonance spectroscopy; ^{1}H NMR, proton magnetic resonance spectroscopy; IR, infrared; TBHP, *tert*-butyl hydroperoxide; TMS, tetramethylsilane.

talum album (Linn.) seed oil (15). Selenium dioxide and 1,4-dioxane were obtained from Aldrich Chemical Company (St. Louis, MO). TBHP (80% solution in di-*tert*-butylperoxide) was purchased from Merck.

General reaction of acetylenic esters with selenium dioxide/TBHP as exemplified by the reaction with methyl undec-10-ynoate (1). A mixture of selenium dioxide (0.57 g, 5.1 mmol), TBHP (2 cm^3), 1,4-dioxane (18 cm^3), and water (2 cm^3) was stirred at room temperature for 30 min. Methyl undec-10-ynoate (**1**, 1.0 g, 5.1 mmol) was added, and the reaction mixture was heated at 85°C for 3 h. Water (60 cm^3) was added, and the reaction mixture was extracted with diethyl ether (3 × 30 cm^3). The ethereal extract was washed with water (20 cm^3) and dried over anhydrous sodium sulfate. The filtrate was evaporated, and the residue was separated by silica gel (60 g) column chromatography by gradient elution using mixtures of *n*-hexane/diethyl ether (100:0, vol/vol, 200 cm^3; 95:5, vol/vol, 200 cm^3; 90:10, vol/vol, 200 cm^3; 80:20, vol/vol, 600 cm^3; 70:30, vol/vol, 600 cm^3; 50:50, vol/vol, 600 cm^3) into two fractions (*viz.* compounds **2** and **3**). The structure of compound **2** (methyl 9-oxo-undec-10-ynoate) (0.1 g, 9%) was determined from the following physical data: R_f = 0.7 (using a mixture of *n*-hexane/diethyl ether, 4:1, vol/vol, as developer); IR (cm^{-1}) 3250 (C-H str., C≡C-H), 2100 (C≡C, str.), 1735 (C=O, str., ester), 1680 (C=O, str., oxo), and 1430; ^{1}H NMR (δ_H) 1.2–1.7 (*m*, 10H, CH_2), 2.28 (*t*, 2H, 2-*H*), 2.58 (*t*, 2H, 8-*H*), 3.22 (*s*, 1H, 11-*H*), and 3.67 (*s*, 3H, COOCH_3); ^{13}C NMR (δ_C) 23.65 (C-7), 24.83 (C-3), 28.67–28.91 (3C, CH_2), 34.02 (C-2), 45.38 (C-8), 51.48 (COOCH_3), 78.37 (C-11), 81.45 (C-10), 174.23 (C-1), and 187.48 (C-9); and the structure of compound **3** (methyl 9-hydroxy-undec-10-ynoate) (0.65 g, 60%) was determined from the following physical data: R_f = 0.3 (using a mixture of *n*-hexane/diethyl ether, 4:1, vol/vol, as developer); IR (cm^{-1}) 3450 (O-H, str.), 3300 (C-H, str., C≡C-H), 2350 (C≡C, str.), 1740 (C=O, str., ester) and 1440; ^{1}H NMR (δ_H) 1.3–1.7 (*m*, 12H, CH_2), 2.31 (*t*, 2H, 2-*H*), 2.46 (*s*, 1H, 11-*H*), 2.99 (*s*, broad, O-*H*), 3.67 (*s*, 3H, COOCH_3) and 4.36 (*t*, 1H, 9-*H*); ^{13}C NMR (δ_C) 24.88 (C-7), 24.96 (C-3), 28.99–29.10 (3C, *C*H2), 34.07 (C-2), 37.56 (C-8), 51.56 (COO*C*H_3), 62.02 (C-9), 72.62 (C-11), 85.25 (C-10), and 174.52 (C-1).

Reaction of methyl octadec-9-ynoate (**4**) with selenium dioxide/TBHP at 85°C for 20 h furnished methyl 8-oxo and 11-oxo-octadec-9-ynoate (**5**, 52 mg, 5%), as determined by the following physical data: R_f = 0.7 (using a mixture of *n*-hexane/diethyl ether, 4:1, vol/vol, as developer); IR (cm^{-1}) 2150 (C≡C, str.) , 1740 (C=O, str., ester) and 1680 (C=O, str., oxo); ^{1}H NMR (δ_H) 0.88 (*t*, 3H, CH_3), 1.2–1.5 (*m*, 14H, CH_2), 1.6–1.9 (*m*, 6H, CH_2), 2.31 (*t*, 2H, 2-*H*), 2.36 (*t*, 2H, -CH_2-C≡C-), 2.50 (*t*, 2H, -C≡C-CO-CH_2-) and 3.66 (*s*, 3H, COOCH_3); ^{13}C NMR (δ_C) 14.10 (C-18), 18.91/18.95 (-C≡C-*C*H_2-), 22.61/22.65 (C-17), 23.92/24.18, 24.73/24.79 (-C≡C-CO-CH$_2$-*C*H_2-), 24.86/24.90 (C-3), 28.88–29.13 (6C, *C*H_2), 31.66/31.81 (C-16), 33.96/34.01 (C-2), 45.40/45.55 (-C≡C-CO-*C*H_2-), 51.45 (COO*C*H_3), 80.91/80.98 (-*C*≡C-CO-), 94.03/94.38 (-C≡*C*-CO-), 174.12/174.16 (C-1), and 188.28/188.53 (-C≡C-*C*O-); methyl 8-hydroxy-11-oxo- and 11-hydroxy-8-oxo-octadec-9-ynoate (**6**, 0.11 g, 10%), as determined from the following physical data: R_f = 0.2 (using a mixture of *n*-hexane/diethyl ether, 4:1, vol/vol, as developer); IR (cm^{-1}) 3450 (O-H, str.), 2900 (C-H, str.), 1740 (C=O, str., ester), and 1675 (C=O, str., oxo); ^{1}H NMR (δ_H) 0.88 (*t*, 3H, CH_3), 1.2–1.7 (*m*, 20H, CH_2), 1.8 (*s*, *br*, O-*H*), 2.31 (*t*, 3H, 2-*H*), 2.56 (*t*, 2H, -C≡C-CO-CH_2-), 3.67 (*s*, 3H, COOCH_3), and 4.52 (*t*, 1H, -C≡C-C*H*OH-); ^{13}C NMR (δ_C) 14.07 (C-18), 22.60/22.64 (C-17), 23.72/23.96 (-C≡C-CHOH-CH$_2$-*C*H_2-), 24.63/24.74 (-C≡C-CO-CH$_2$-*C*H_2-), 24.83/25.06 (C-3), 28.46–29.71 (4C, *C*H_2), 31.65/31.76 (C-16), 33.94/34.01 (C-2), 36.89/36.99 (-C≡C-CHOH-*C*H2-), 45.23/45.44 (-C≡C-CO-*C*H_2-), 51.53/51.57 (COO*C*H_3), 62.12/62.19 (-C≡C-*C*HOH-), 83.46/92.64 (-*C*≡C-) 174.14 (C-1), and 187.91 (-C≡C-*C*O-); and one fraction containing pure methyl 8,11-dihydroxy-octadec-9-ynoate (**7**, 0.27 g, 24%) as determined from the following physical data: R_f = 0.1 (using a mixture of *n*-hexane/diethyl ether, 4:1, vol/vol, as developer); IR (cm^{-1}) 3400 (O-H, str.), 1740 (C=O, str., ester) and 1460; ^{1}H NMR (δ_H) 0.88 (*t*, 3H, CH_3), 1.2–1.7 (*m*, 22H, CH_2), 2.31 (*t*, 2H, 2-*H*), 2.5 (*s*, *br*, O-*H*), 3.67 (*s*, 3H, COOCH_3), and 4.39 (*t*, 2H, -C≡C-C*H*OH-); ^{13}C NMR (δ_C) 14.10 (C-18), 22.59 (C-17), 24.77 (-C≡C-CHOH-CH$_2$-*C*H_2-), 24.88 (C-3), 28.79–29.23 (4C, *C*H_2), 31.81 (C-16), 34.03 (C-2), 37.57/37.76 (-C≡C-CHOH-*C*H_2-), 51.54 (COO*C*H_3), 62.34/62.40 (-C≡C-*C*HOH-), 85.81/86.04 (-*C*≡*C*-), and 174.40 (C-1).

Reaction of methyl octadeca-6,8-diynoate (**8**) with selenium dioxide/TBHP at 85°C for 20 h furnished a mixture of methyl 5-oxo- and 10-oxo-octadeca-6,8-diynoate (**9**, 0.13 g, 12%) as determined from the following physical data: R_f = 0.6 (using a mixture of *n*-hexane/diethyl ether, 4:1, vol/vol, as developer); IR (cm^{-1}) 1740 (C=O, str., ester) and 1675 (C=O, str., oxo) ; ^{1}H NMR (δ_H) 0.88 (*t*, 3H, CH_3), 1.2–1.9 (*m*, 14H, CH_2), 2.3–2.5 (*m*, 6H, CH_2), 2.55, and 2.65 (*t*, 2H, -C≡C-C≡C-CO-CH_2-) and 3.68 (*t*, 3H, COOCH_3); 13C NMR (δ_C) 14.42 (C-18), 19.74/19.97 (-C≡C-C≡C-CH$_2$-), 22.99 (C-17), 24.30 (-C≡C-C≡C-CO-CH$_2$-*C*H_2-), 24.31/24.35 (C-3), 27.51–29.72 (5C, *C*H_2), 32.11/32.17 (C-16), 33.04/33.67 (C-2), 44.67/45.83 (-C≡C-CO-*C*H_2-), 51.93 (COO*C*H_3), 64.08/64.56 (-C≡C-*C*≡C-CO-), 72.31/ 72.73 (-C≡*C*-C≡C-CO-), 76.14/76.87 (-*C*≡C-C≡C-CO-), 89.96/91.39 (-C≡C-C≡*C*-CO-), 173.57/173.89 (C-1) and 186.35/187.63 (-C≡C-C≡C-*C*O-).

Reaction of methyl octadec-11*E*-en-9-ynoate (**10**) with selenium dioxide/TBHP at 75°C for 20 h gave two fractions: *viz.* methyl 8-oxo-octadec-11(*E*)*Z*-en-9-ynoate (**11**, 60 mg, 6%) as determined from the following physical properties: R_f = 0.6 (using a mixture of *n*-hexane/diethyl ether, 4:1, vol/vol, as developer); IR (cm^{-1}) 2200 (C≡C, str.), 1740 (C=O, str. ester), 1670 (C=O, str. oxo), 1460 and 1420; ^{1}H NMR (δ_H) 0.89 (*t*, 3H, CH_3), 1.2–1.7 (*m*, 16H, CH_2), 2.19 (*dt*, *J* = 6.5 Hz, 2H, 13-*H*), 2.30 (*t*, 2H, 2-*H*), 2.56 (*t*, 2H, 7-*H*), 3.66 (*s*, 3H, COOCH_3), 5.63 (*d*, 1H, 11-*H*) and 6.28/6.47 (*m*, 1H, 12-*H*); ^{13}C NMR (δ_C) 14.07 (C-18), 22.57 (C-17), 23.95 (C-6),

24.73 (C-3), 28.23–29.03 (4C, CH_2), 31.01/31.50 (C-13), 31.60 (C-16), 33.94/34.05 (C-2), 45.24/45.40 (C-7), 51.43 ($COOCH_3$), 87.01/87.86 (C-9), 90.20/92.06 (C-10), 107.04/107.55 (C-11), 151.34/152.72 (C-12), 174.09 (C-1), and 187.87/187.97 (C-8); high-resolution mass spectrum: M^+ found 306.2195 (calc. for $C_{19}H_{30}O_3$, 306.2189), low-resolution mass spectral result [*m/z* (relative intensity)]: 275 (9.3), 221 (10.5), 217 (7.9), 178 (18.1), 172 (9.8), 171 (100), 164 (20.8), 163 (76.3), 149 (21.9), 139 (24.8), 135 (12.6), 121 (17.5), 111 (18.4), 107 (20.3), 105 (10.3), 95 (12.6), 94 (22.4), 93 (20.5), 91 (17.4), 83 (12.5), 81 (13.4), 79 (37.4), 77 (14.5), 73 (17.1), 69 (16.0) and 67 (12.1); and methyl 8-hydroxy-octadec-11*E*-en-9-ynoate (**12**, 0.74 g, 70%, 90% pure by NMR) as determined from the following physical data: R_f = 0.3 (using a mixture of *n*-hexane/diethyl ether, 4:1, vol/vol, as developer); IR (cm^{-1}) 3450 (O-H, str.), 1740 (C=O, str., ester), 1460 and 1440; ^1H NMR (δ_H) 0.88 (*t*, 3H, CH_3), 1.2–1.8 (*m*, 18H, CH_2), 2.08 (*dt*, *J* = 6.7 Hz, 2H, 13-*H*), 2.31 (*t*, 3H, 2-*H*), 2.40 (*s*, *br.*, O-H), 3.66 (*s*, 3H, $COOCH_3$), 4.48 (*t*, 1H, C*H*OH-), 5.48 (*d*, 1H, 11-*H*), and 6.16 (*m*, 1H, 12-*H*); ^{13}C NMR (δ_C) 14.10 (C-18), 22.60 (C-17), 24.86 (C-3), 25.02 (C-6), 28.65–29.03 (4C, CH_2), 31.66 (C-13), 31.79 (C-16), 34.06 (C-2), 37.82 (C-7), 51.51 ($COOCH_3$), 62.76 (C-8), 83.61 (C-10), 88.62 (C-9), 108.85 (C-11), 145.41 (C-12), and 174.34 (C-1).

RESULTS AND DISCUSSION

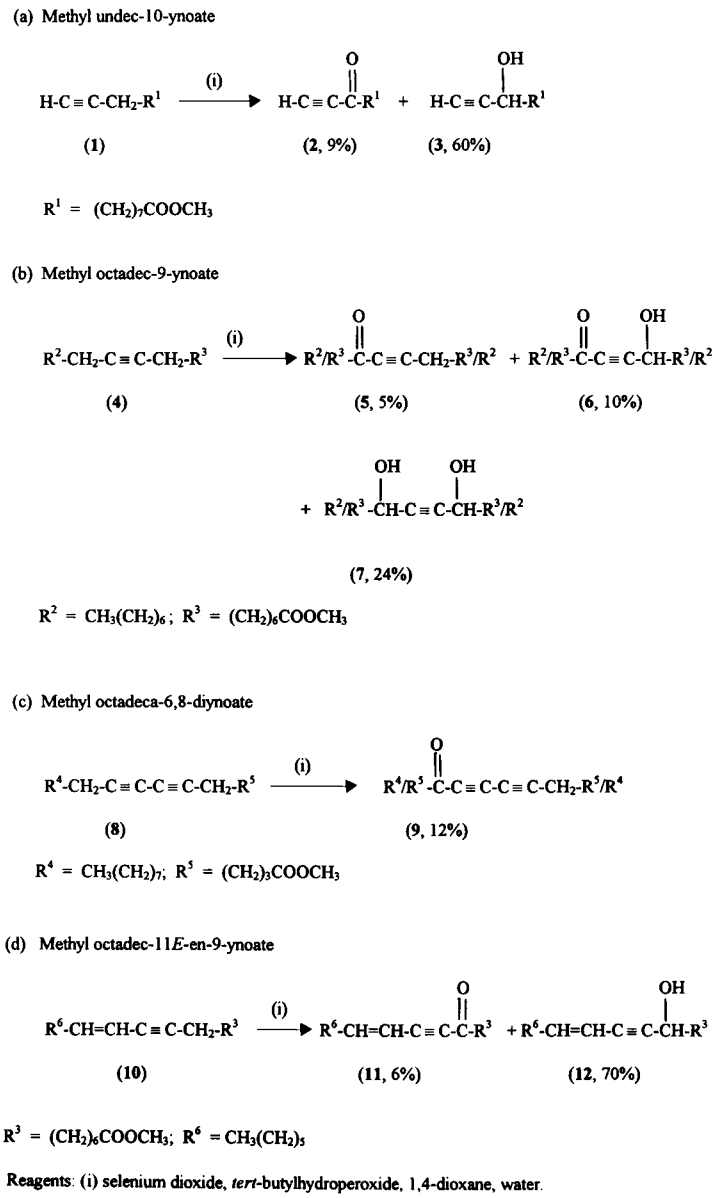
Selenium dioxide hydroxylation reactions of olefins are normally conducted in dichloromethane as the solvent (4). However, when methyl undec-10-ynoate (**1**) was stirred with SeO_2/TBHP in dichloromethane for 48 h as recommended, a complex mixture of products consisting of unreacted substrate, hydroxylated, and other polar oxygenated products was obtained. The polar products could not be separated into individual components by silica gel chromatography. A possible reason for obtaining such an intractable mixture of polar compounds is the absence of water in the system, which may cause attack of the unsaturated system in addition to propargylic hydroxylation of the methylene group adjacent to the acetylenic bond. In order to obtain a cleaner reaction product from such a reaction, various organic solvents or mixtures of solvents were tried. A mixture of dioxane and water (9:1, vol/vol) was found to offer the best reaction condition. Hydroxylation reactions were thus carried out in aqueous dioxane with methyl undec-10-ynoate (**1**), methyl octadec-9-ynoate (**4**), methyl octadeca-6,8-diynoate (**8**), and methyl santalbate (methyl octadec-11*E*-en-9-ynoate, **10**) as presented in Scheme 1.

Reaction of methyl undec-10-ynoate (**1**) with SeO_2/TBHP in aqueous dioxane gave two products. The less polar product was identified as methyl 9-oxo-undec-10-ynoate (**2**, 9%) from the IR absorption bands at 3250, 2100 (terminal acetylenic system), 1680 (C=O, oxo) cm^{-1} and confirmed by the shift of the acetylenic proton at δ_H 3.22 (*s*, 1H, -C≡C-*H*) and the following carbon shifts δ_C: 81.45 (C-10), 78.37 (C-11), 187.48 (C-9), 45.38 (C-8), and 23.65 (C-7). The shifts of the C-11 carbon atom were further confirmed by two-dimensional ^1H-^{13}C COSY correlation NMR spectroscopic technique. The polar and major fraction was found to be the hydroxylated product, methyl 9-hydroxy-undec-10-ynoate (**3**, 60%) as characterized by the IR absorption bands at 3450 (O-H), 3300 (-C≡C-H), 2350 (C≡C) cm^{-1} and supported by the shifts of the protons at δ_H 2.46 (*s*, 1H, -C≡C-*H*) and 2.99 (*s*, *br*, 1H, O-*H*) and carbon shifts at δ_C 72.62 (C-11), 85.25 (C-10), 62.02 (C-9), 37.56 (C-8), and 24.88 (C-7) ppm. The appearance of compound **2** in the reaction product showed that hydroxy-acetylenic ester **3** was further oxidized by SeO_2/TBHP.

Treatment of methyl octadec-9-ynoate (**4**) with SeO_2/TBHP under similar experimental conditions gave, on careful silica gel column chromatographic separation, three fractions containing mixtures of positional isomers of oxygenated acetylenic fatty esters: *viz.* methyl 8-oxo- and 11-oxo-octadec-9-ynoate (**5**, 5%), methyl 8-hydroxy-11-oxo- and 11-hydroxy-8-oxo-octadec-9-ynoate (**6**, 10%) and a fraction of pure dihydroxy-acetylenic fatty ester (methyl 8,11-dihydroxy-octadec-9-ynoate, **7**, 24%). The presence of two positional isomers in the isolated fractions (namely, compounds **5** and **6**) was apparent from the ^{13}C NMR spectra of these products, which showed twin signals (about 1:1 ratio in signal intensity) for some of the critical carbon signals.

The presence of a conjugated oxo-acetylenic system in compound **5** was evident from the IR absorption bands at 1680 (C=O) and 2150 (C≡C) cm^{-1} and was confirmed by the shifts of the following carbon atoms: δ_C 94.03/94.38 (-C≡*C*-CO-), 80.91/80.98 (-*C*≡C-CO-), 188.28/188.53 (-C≡C-*C*O-), 45.40/45.55 (-C≡C-CO-CH_2-), 18.91/18.95 (CH_2 -C≡C-CO-). The structures of the positional isomers of the hydroxy-keto derivatives **6** were identified by the absorption bands for the O-H (3450 cm^{-1}) and C=O (keto, 1675 cm^{-1}) vibrations in the IR spectrum and confirmed by ^1H NMR analysis: δ_H 4.52 [*t*, 1H, -CO-C≡C-C*H*(OH)-] and from the ^{13}C NMR analysis: δ_C 187.91 [-*C*O-C≡C-CH(OH)-], 83.46/92.64 [-CO-*C*≡*C*-CH(OH)-] and 62.12/62.19 [-CO-C≡C-*C*H(OH)-]. The ^{13}C NMR spectral analysis of the dihydroxy-acetylenic derivative **7** gave a "clean" spectrum with characteristic shifts at δ_C 85.81/86.04 for the shifts of the acetylenic carbon atoms [-(OH)CH-*C*≡*C*-CH(OH)-] and signals at δ_C 62.34/62.40 for the hydroxy-bearing methine carbon atoms [-(OH)-*C*H-C≡C-*C*H(OH)-]. Signals corresponding to the shifts of the methylene carbon atoms adjacent to the acetylenic bond were absent, which confirmed that these α-methylene groups were hydroxylated.

When methyl octadeca-6,8-diynoate (**8**) was reacted with SeO_2/TBHP in aqueous dioxane, a very complex mixture of polar oxidized derivative was obtained, which appeared as a continuous streak on a thin-layer chromatographic plate upon analysis. Separation of the reaction products by silica gel column chromatography permitted only a nonpolar fraction to be purified. This fraction comprised a mixture of methyl 5- and 10-oxo-octadeca-6,8-diynoate (**9**, 12%). These positional isomers gave a characteristic absorption band at 1675 cm^{-1}



SCHEME 1

for the C=O stretching vibration. The conjugated oxo-diacetylenic system (-CO-C≡C-C≡C-) was identified from the data of the ^{13}C NMR spectrum: δ_C 186.35/187.63 (-*C*O-C≡C-C≡C-) and by the four pairs of carbon signals at δ_C 64.08/64.56, 72.31/72.73, 76.14/76.87, and 89.96/91.39 due to the shifts of the diacetylenic carbon atoms. While no hydroxy-diacetylenic ester derivatives could be separated from the products of this reaction, the identification of compound **9** as a mixture of oxo-diacetylenic ester derivatives implied that the corresponding hydroxy-diacetylenic ester derivatives must have been initially formed during the reaction between the diacetylenic substrate and SeO_2/TBHP. Attempts to separate the complex polar fractions of this reaction as acetoxy derivatives by chromatography were unsuccessful.

Reaction of methyl santalbate (methyl octadec-11*E*-en-9-ynoate, **10**), which contains a conjugated enyne system in the alkyl chain, with SeO_2/TBHP in aqueous dioxane furnished a mixture of methyl 8-oxo-octadec-11(*E*)*Z*-en-9-ynoate (**11**, 6%) and methyl 8-hydroxy-octadec-11*E*-en-9-ynoate (**12**, 70%, 90% pure by NMR). Hydroxylation of methyl santalbate appeared to be regiospecific with the hydroxylation taking place only at the methylene carbon adjacent to the acetylenic bond to give a propargylic system. No product corresponding to an allylic hydroxy derivative, i.e., hydroxylation at the methylene carbon adjacent to the olefinic bond, was found. Compound **11** was characterized by the absorption bands at 1670 and 2200 cm^{-1} (α-oxo-acetylenes) and from the ^{13}C NMR spectral analysis: δ_C 31.01/31.50 (C-13), 45.24/45.40 (C-7), 87.01 /87.86 (C-9), 90.20/92.06 (C-10), 107.04/107.55 (C-11), 151.34/152.72 (C-12), and 187.87/187.97 (C-8). The presence of a signal at δ_C 31.01/31.58 due to the shift of the C-13 carbon atom indicated

that hydroxylation had not taken place at the α-methylene adjacent to the olefinic bond. This fact was also supported by the absence of a signal at δ_C 18-19 (shift region for a methylene carbon adjacent to an acetylenic bond). The high-resolution mass spectral analysis of compound **11** gave a molecular ion M^+ = 306.2195, which agreed closely with the calculated molecular mass (calc. 306.2189). From the low-resolution mass spectrum, the base peak at *m/z* 171 (100% intensity) and the ion fragment at *m/z* 163 (76.3% intensity) identified the position of the oxo function at the C-8 position of the alkyl chain. These peaks resulted from the α-cleavage of the C-C bonds adjacent to the C=O function.

The structure of compound **12** (methyl 8-hydroxy-octadec-11*E*-en-9-ynoate) was established on the basis of the following spectral data. The IR spectrum of this compound showed a broad and strong band at 3450 cm^{-1} for the stretching vibration of the hydroxy function. The ^1H NMR spectrum showed double triplet (J = 6.7 Hz) δ_H 2.08 due to the shifts of the allylic protons (13-*H* , -C*H*$_2$-CH=CH-C≡C-), and a triplet at δ_H 4.48 was due to the shift of the methine proton (8-*H*) bearing the hydroxy group. The doublet and multiplet at δ_H 5.48 and 6.16 were the result of the shifts of the 11-*H* and 12-*H* protons, respectively. The ^{13}C NMR spectrum of compound **12** showed a signal at δ_C 62.76 for the methine carbon atom of the *C*HOH system. The appearance of a signal for the C-13 carbon atom (δ_C 31.66) indicated that hydroxylation had not taken place at the α-methylene adjacent to the olefinic bond. This fact was also supported by the absence of a signal at δ_C 18-19 region for the shift of a methylene carbon atom adjacent to the acetylenic bond of the enyne system of methyl santalbate (17). A possible explanation for the regiospecific hydroxylation of the enyne system at the methylene adjacent to the triple bond is likely due to the stronger electron withdrawing property of the acetylenic bond (sp^1 character of the acetylenic carbon atoms) than that of the olefinic bond, which permits propargylic hydroxylation to proceed faster than the allylic hydroxylation. Also, the spectra and gas chromatographic analysis of the unreacted material obtained after the oxidation reaction revealed the presence of *Z*-isomer which suggests the isomerization of *E*-double bond of 1,3-enyne system to its *Z*-analog during the treatment with selenium dioxide. This observation is unprecedented and the isomerization of the double bond from the *E*- to the *Z*-double bond of the enyne system may be due to the catalytic effect of selenium metal generated from the hydroxylation process.

It can be concluded from these results that hydroxylation of methylene groups adjacent to an acetylenic bond was achieved when the acetylenic fatty ester substrates were reacted with SeO_2/TBHP in aqueous dioxane. Some of the hydroxy groups in these derivatives were also oxidized to the corresponding oxo functions. However, in the case of a conjugated olefin-acetylenic (enyne) system, the hydroxylation reaction was regiospecific with hydroxylation taking place at the methylene carbon atom adjacent to the acetylenic bond only. Thus, α-hydroxylation of methyl santalbate with SeO_2/TBHP gave exclusively methyl 8-hydroxy-octadec-11*E*-en-9-ynoate in 70% yield and 6% of the corresponding oxo derivative.

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ARTICLE

The Effect of Dietary Docosahexaenoic Acid on Platelet Function, Platelet Fatty Acid Composition, and Blood Coagulation in Humans

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ABSTRACT: The effect of dietary docosahexaenoic acid (DHA) in the absence of eicosapentaenoic acid (EPA) has been studied infrequently in humans under controlled conditions. This 120-d study followed healthy, adult male volunteers who lived in the metabolic research unit (MRU) of the Western Human Nutrition Research Center for the entire study. The basal (low-DHA) diet consisted of natural foods (30 en% fat, 15 en% protein, and 55 en% carbohydrate), containing <50 mg/d of DHA, and met the recommended daily intake for all essential nutrients. The high-DHA (intervention) diet was similar except that 6 g/d of DHA in the form of a triglyceride containing 40% DHA replaced an equal amount of safflower oil in the basal diet. The subjects (ages 20 to 39) were within −10 to +20% of ideal body weight, nonsmoking, and not allowed alcohol in the MRU. Their exercise level was constant, and their body weights were maintained within 2% of entry level. They were initially fed the low-DHA diet for 30 d. On day 31, six subjects (intervention, group A) were placed on the high-DHA diet; the other four subjects (controls, group B) remained on the low-DHA diet. Platelet aggregation in platelet-rich plasma was determined using ADP, collagen, and arachidonic acid. No statistical differences could be detected between the amount of agonist required to produce 50% aggregation of platelet-rich plasma before and after the subjects consumed the high-DHA diet. The prothrombin time, activated partial thromboplastin time, and the antithrombin-III levels in the subjects were determined, and, again, there were no statistically significant differences in these three parameters when their values were compared before and after the subjects consumed the high-DHA diet. In addition, the *in vivo* bleeding times did not show any significant difference before and after the subjects consumed the high-DHA diet (9.4 ± 3.1 min before and 8.0 ± 3.4 min after). Platelets from the volunteers exhibited more than a threefold increase in their DHA content from 1.54 ± 0.16 to 5.48 ± 1.21 (wt%) during the DHA feeding period. The EPA content of the subjects' platelets increased from 0.34 ± 0.12 to 2.67 ± 0.91 (wt%) during the high-DHA diet despite the absence of EPA in the subjects' diets. The results from this study on blood clotting parameters and *in vitro* platelet aggregation suggest that adding 6 g/d of dietary DHA for 90 d to a typical Western diet containing less than 50 mg/d of DHA produces no observable physiological changes in blood coagulation, platelet function, or thrombotic tendencies in healthy, adult males.

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Since the reports of Bang and Dyerberg (1–3) in the 1970s concerning the health and diets of indigenous Greenland natives, there has been intense interest in the health effects of dietary n-3 polyunsaturated fatty acids, particularly those with 20- and 22-carbon chain lengths and five or more double bonds. Bang and Dyerberg's observations immediately prompted human feeding studies (4–7) with marine oils. It was found that long-chain polyunsaturated n-3 fatty acids decrease plasma triacylglyceride levels (8,9) and caused a prolongation in bleeding times and a decrease in platelet aggregation to various agonists *in vitro* (10–12). Almost all the initial experimental feedings studies (5,6,8–12) used marine oils containing various proportions of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). It was often assumed that EPA was the physiologically active fatty acid in the oil that altered the metabolic pathways and hemostatic parameters measured in these experiments. For several years there has been controversy about the physiological consequences of dietary DHA contained in marine oils (13). Investigators using EPA concentrates were able to show that EPA alone would lower plasma triglycerides and prolong bleeding times (14–16). There was less interest in DHA, and no convenient source of purified DHA was available in sufficient quantity to use in human feeding studies.

Von Schacky and Weber (16) in 1985, using either purified EPA or DHA, reported that both compounds reduced platelet aggregation *in vitro*. They concluded that DHA is an important factor in the reduction of *in vitro* platelet aggregation when fish oils are consumed by humans. Still, this was a short-term study (6 d) with only seven volunteers, who consumed EPA and DHA as ethyl esters rather than triglycerides.

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Abbreviations: APTT, activated partial thromboplastin time; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MRU, metabolic research unit; PRP, platelet-rich plasma; PT, prothrombin time.

The physiological effects of dietary DHA are complicated, because as shown by several investigators (16–18), DHA is retroconverted to EPA *in vivo* by the "Sprecher shunt" (19, 20). Thus, even if one feeds only DHA to human volunteers, it is difficult to determine whether observed physiological changes are due to DHA or to EPA produced by retroconversion of dietary DHA. Still, we felt that it was important to test whether DHA in longer-term human feeding studies had the same physiological effects as reported for EPA or fish oils containing both EPA and DHA, particularly if the DHA could be fed in the form of a triglyceride.

Recently a purified triacylglycerol oil, produced by a selected algal strain, containing 40% DHA and no EPA has become available in sufficient quantities to conduct human feeding studies (DHASCO™; Martek Biosciences Corp., Columbia, MD). This study was designed to determine if the physiological and biochemical changes reported in humans consuming marine oils containing both EPA and DHA or EPA alone can be reproduced by a diet containing only DHA. This paper reports the results of this study on platelet function *in vitro*, bleeding times, coagulation factors, and platelet fatty acid composition from a human feeding study in which a group of normal healthy volunteers consumed a diet containing 6 g/d of DHA and no EPA for 90 d after a 30-d stabilization period during which they consumed a diet containing less than 50 mg/d DHA.

STUDY DESIGN

The study was designed as a longitudinal, single-blind feeding study in which 12 healthy volunteers lived in the metabolic research unit (MRU) of the Western Human Nutrition Research Center for 120 d. All subjects were fed a stabilization diet (low-DHA) for 30 d after they entered the MRU. On day 31 of the study, 8 of the subjects, designated Group A, were switched to a diet (high-DHA) containing 6 g/d of DHA (from 15 g of DHASCO™ oil). The remaining 4 subjects, designated Group B, continued to consume the low-DHA diet, which contained less than 50 mg/d of DHA. Two subjects dropped out of the study, both from Group A. The subjects were discharged from the MRU on day 120 of the study. Figure 1 shows a schematic plan of the study design and the times of the blood draws.

The protocol for this study was approved by the Institutional Review Boards of the University of California at Davis and the USDA, Houston, TX. The subjects were fed the low-DHA diet, containing about 30% of calories as fat for 30 d, then divided into two groups. Group A (n = 6) was switched to a high-DHA diet containing 6 g/d of DHA (15 g/d of DHASCO™ oil). They remained on this diet for 90 d. Group B (n = 4) continued on the low-DHA diet.

At the beginning of the study, each participant's caloric intake was estimated and the caloric content of the meals calculated to maintain current body weight. As the estimates were subject to error, the participants were weighed every day and the caloric value of their meals adjusted occasionally, if necessary, to maintain their weight within the limits of the study, i.e., ±2% of their weight upon entry into the study. Thus, the average weights of the participants did not vary significantly during the 120 d of the study. If all the intakes for each volunteer were averaged during the entire study, the mean energy intake was approximately 2800 Kcal/d.

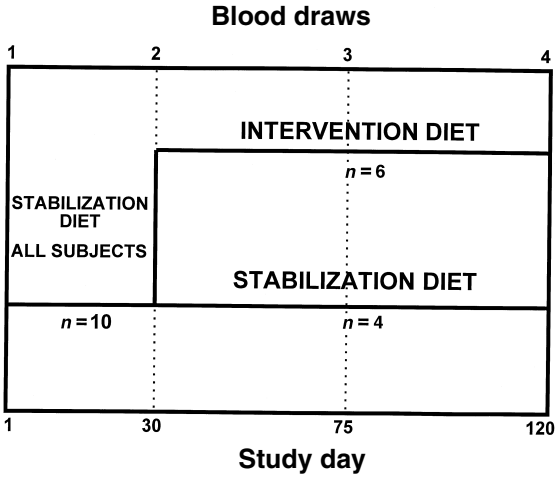

FIG. 1. Schematic plan of the design for this feeding study showing the days of the blood draws and dietary periods. Note that the values were compared for the intervention group (A) after 30 d on the stabilization diet to that obtained after 90 d on the intervention diet.

Diets. Except for DHA supplement in the form of the DHASCO™ oil and a vitamin E supplement, the diets consisted of natural foods. The macronutrient composition of the diets is given in Table 1. (A complete description of the diets, listing all the major and minor nutrients, is available upon request.) A 5-d menu cycle was used throughout the study.

TABLE 1
Macronutrient Composition of Diets: Proximal Analysis[a]

	Intervention diet, high DHA		Stabilization diet, low DHA	
	Measured	Target value	Measured	Target value
	Total calories (%)			
Macronutrient energy distribution				
Protein	15.2	15.0	14.6	15.0
Fat	30.9	30.0	29.0	30.0
Carbohydrate	53.9	55.0	56.4	55.0
Cholesterol content: mean, mg/day	—	360	—	360
Fatty acid energy distribution				
Saturated	8.9	8	8.1	8
Monounsaturated	9.2	10	9.2	10
Polyunsaturated, n-6	6.5	7	8.5	8.5
Polyunsaturated, n-3	2.8	3	1.1	1.5
Trans	1.9	2	2.1	2.0
P/S ratio	1.2	1.0	1.1	1.0

[a]Abbreviations: DHA, docosahexaenoic acid; P/S ratio, polyunsaturated fatty acid/saturated fatty acid ratio.

TABLE 2
Fatty Acid Composition (wt%) of Experimental Diets

Fatty acid methyl ester	Intervention diet, high DHA[a]	Stabilization diet, low DHA[a]
12:0 (Laurate)	1.3 ± 0.3	0.7 ± 0.3
14:0 (Myristate)	4.3 ± 0.7	2.2 ± 0.8
16:0 (Palmitate)	16.6 ± 1.6	16.3 ± 1.6
16:1n-9	0.7 ± 0.4	0.9 ± 0.4
18:0 (Stearate)	7.1 ± 1.0	7.5 ± 0.9
18:1*t*: All isomers	6.2 ± 1.0	7.0 ± 1.0
18:1n-9(Oleate)	26.6 ± 1.8	26.0 ± 1.7
18:1n-7	1.7 ± 0.3	2.0 ± 0.2
18:1n-5	1.5 ± 0.4	2.1 ± 0.4
18:2*tt* and 19:0	0.5 ± 0.1	0.6 ± 0.1
18:2n-6 (Linoleate)	21.6 ± 2.7	28.3 ± 2.2
18:3n-3 (α-Linolenate)	2.6 ± 0.4	3.2 ± 0.4
22:0 (Behenate)	0.2 ± 0.0	0.2 ± 0.0
20:5n-3 (Eicosapentaenoate)	0.4 ± 0.1	0.3 ± 0.1
22:6n-3 (Docosahexaenoate)	6.5 ± 0.5	>0.1 ± 0.1
Total	98.0 ± 0.4	97.2 ± 0.3
Unknowns	2.0 ± 0.4	2.8 ± 0.3

[a]Values given as means ± SD (n = 5). For abbreviation see Table 1.

Proximate analysis was made on five individual diet composite samples taken from each menu once during the study for both the stabilization and intervention diets. The results for the five composite samples were averaged to find the actual composition of the diets. No alcohol was included in these diets. The nutrient composition of the diets was calculated from a computerized nutrient data bank using the data from USDA Handbook 8 (21) and adjusted to provide at least the recommended daily intake for known essential nutrients (22). A supplement of 20 mg/d of d-α-tocopherol [Vitamin E (obtained from Bronson Pharmaceutical, St. Louis, MO), 100-mg capsules given every 5 d] was included in the diet to ensure adequate antioxidant levels in the volunteers during the study.

Table 2 gives the measured fatty acid composition of both diets. Inspection of Table 2 indicates that all the major fatty acids were very similar except that the level of DHA was elevated in the high-DHA diet and the level of linoleic acid was reduced.

MATERIALS AND METHODS

The fatty acid composition of the DHASCO™ oil is given in Table 3. The oil also contained 0.025% α-tocopherol and 0.025% ascorbyl palmitate. The oil was kept under nitrogen and at −20°C until it was needed for food preparation. Open bottles were flushed with N_2 before they were resealed and returned to the refrigerator. Since the DHA was fed in a cold yogurt cocktail once daily, it is unlikely that any oxidative degradation took place between the time when the food was prepared and it was eaten. The control group also consumed a yogurt cocktail containing safflower oil once daily.

Subjects. The physical characteristics of the subjects on entry into the study (Study Day 0) are given in Table 4. Initially 12 volunteers were included in the study, but two were unable to complete the protocol; both were from intervention Group A. Thus, at the end of the study, Group A had six volunteers and the control, Group B, had four. See the section on statistical analyses below for information on the analyses of the data from the two groups.

TABLE 3
Fatty Acid Composition of DHASCO™ Oil[a]

Fatty acid methyl ester	Wt%
11:0	0.46 ± 0.02
12:0 (Laurate)	3.84 ± 0.07
14:0 (Myristate)	13.48 ± 0.08
16:0 (Palmitate)	11.51 ± 0.02
16:1n-9	1.39 ± 0.01
18:0 (Stearate)	1.15 ± 0.01
18:1n-9 (Oleate)	27.88 ± 0.18
18:2n-6 (Linoleate)	1.02 ± 0.01
22:0 (Behenate)	0.30 ± 0.01
22:6n-3 (Docosahexaenoate)	39.01 ± 0.01
Total	100.04 ± 0.03[b]

[a]DHASCO™ oil produced by Martek Biosciences Corporation, Columbia, MD. Analysis was performed by USDA procedures.
[b]Total exceeds 100% due to rounding.

Measurements. Blood was drawn according to the schedule shown in Figure 1 between 7:00 and 8:00 a.m. after an overnight fast. It was drawn into vacutainers with appropriate anticoagulants using a Teflon catheter (Angiocath; Deseret Medical, Sandy, UT) for fatty acid analysis or into syringes containing 1 mL of citrate solution for platelet aggregation studies (23). Platelet-rich plasma (PRP) was prepared by low-speed centrifugation for 10 min at 100 × g. Platelet-poor plasma was prepared by respinning the blood after removal of the PRP for 15 min at 400 × g (24).

Platelet aggregation. Solutions of the aggregating agents were prepared as recommended by Chrono-log Corp. (Havertown, PA) as follows: ADP, 1 μM/μL; collagen, 0.5 μg/mL; arachidonic acid, 0.05 μM/μL. The *in vitro* aggregation characteristics of the subjects' platelets were measured in a Chrono-log Model 560 dual channel aggregometer. An aggre-

TABLE 4
Characteristics of Subjects[a]

Parameter	Values	
	Intervention group	Control group
Number of subjects completing study	6	4
Age: Yr (mean ± SD)	33.1 ± 4.8	33.3 ± 6.1
Weight: kg (mean ± SD)	78.6 ± 11.0	74.7 ± 8.4
Body mass index: kg/M^2 (mean ± SD)	23.7 ± 2.0	23.1 ± 2.3
Blood pressure: systolic (mean ± SD)	128.0 ± 17.4	118.8 ± 7.5
Blood pressure: diastolic (mean ± SD)	75.5 ± 7.6	69.3 ± 7.6
Bleeding times: min (mean ± SD)	7.6 ± 2.8	6.4 ± 1.2

[a]Sex, all male; smokers, none.

gation testing methodology description has been published elsewhere (24). The threshold for aggregation was determined when the amount of a particular reagent produced at least 50% of maximum aggregation within 15 min of its addition to the PRP. PRP preparations were all adjusted to 300,000 platelets/mm^3. All aggregation tests were performed within 4 h of the initial blood draw.

Platelet counts. Platelet counts were determined with an electronic particle counter, a Baker-Serono Model 9000 Differential Cell Counter (Allentown, PA) electronic particle counter and cytometer using the manufacturer's recommended procedures (Operations Manual, System 9000 Differential Cell Counter, Baker-Serono Diagnostics, Inc., Allentown, PA, 1988, Manual No. DS-014A) (25).

Bleeding times. Bleeding times were measured on the left or right proximal forearm of each subject using a 1-mm deep by 1-cm long incision using a Simplate II bleeding time device (Organon Teknika, Durham, NC). A wipe of the incision was made every 30 s. Time was measured with a hand-held stopwatch (24).

Clotting factors. Prothrombin time (PT) and the activated partial thromboplastin times (APTT) were measured by the methods described by Houghie (24). Briefly, platelet poor plasma was prepared as described elsewhere (24), and the PT and APTT were measured using the Bio/Data Corporation Model MCA 110 instrument (Bio/Data Corporation, Hatboro, PA) with Organon Teknika coagulation reagents.

Lipid extraction and fatty acid analysis. Platelets were extracted by the procedures described by Nelson (27,28) using chloroform/methanol (2:1, vol/vol). Samples were then transmethylated with methanolic HCl (7%, w/w) by the procedures described elsewhere (29,30). When the total lipid extract was transmethylated, the impurities, extracted into the hexane phase after termination of the reaction, were removed by thin-layer chromatography as described by Nelson (28). Because the amount of material available from human platelets for fatty acid analysis was limited, fatty acid methyl esters were obtained by direct transmethylation of the intact tissue without prior solvent extraction as described in an earlier publication (31). The conditions of the capillary gas–liquid chromatography have been described elsewhere (29). The gas–liquid chromatographic column was a 30-m by 0.25-mm fused-quartz column coated with SP-2340 purchased from Supelco, Inc. (Bellefonte, PA). The gas–liquid chromatographic data were processed with Hewlett-Packard ChemStation software (Palo Alto, CA) running on an IBM-compatible desktop computer.

Statistical analysis. The clotting data and aggregation data were analyzed using the paired *t*-test with $n = 6$ for the intervention group (A) or $n = 4$ for the control group (B), or analysis of variance with repeated measures. The subjects acted as their own controls; statistically significant differences were determined by comparing values of the various parameters on study days 30 (baseline) and 120 (end of study). Because the small number of subjects in the control made the chances of detecting significant changes in that group unlikely, no statistical comparisons were made between the control group and the intervention group. The control group was included to detect temporal drift while the volunteers were confined to the MRU. The fatty acid data were analyzed with a single-tailed t-test. All the statistical analyses were performed with the PC SAS data system, version 6.11, obtained under license from the SAS Institute PC-SAS (SAS PC Manual; SAS Institute, Chapel Hill, NC, 1993).

RESULTS

In this study very few physiological changes in the blood clotting systems, platelet function, or the platelet fatty acid composition were detected in subjects who consumed DHA at the level of 6 g/d for 90 d. The average bleeding times for the six subjects after consuming the high-DHA diet for 90 d were 8.00 ± 3.36 min compared to a baseline value of 9.36 ± 3.06 min on study day 30 (they had consumed the low-DHA diet for 30 d). Thus, in these six subjects the bleeding time was actually less after consuming DHA, but the difference between these values did not reach statistical significance ($P = 0.06$). Bleeding time values for the four control subjects were 5.88 ± 0.25 min at 30 d and 6.38 ± 1.89 min at 120 d. Red blood cell counts, hemoglobin levels, and the average hematocrit did not change significantly in either group during the study. Blood pressure, which was measured daily in all volunteers, did not change significantly from the baseline values in either the intervention or control groups throughout the study.

Table 5 lists the platelet aggregation *in vitro* and the platelet counts determined after 90 d on high-DHA diets and 30 d on the low-DHA diet for the 10 subjects in this study. The platelet counts are the actual platelet numbers in whole blood of the volunteers, not the adjusted values used for the aggregation tests. Again, there were no statistically significant changes in either the aggregation values or the platelet

TABLE 5
Platelet Aggregation and Cell Count

	Study day		
	30 Low-DHA diet[a]	120 High-DHA diet[a]	*P*-value
Intervention group ($n = 6$)			
ADP (μM)	2.17 ± 0.89	2.67 ± 1.33	0.10
Collagen (μg/mL)	0.45 ± 0.07	0.43 ± 0.06	0.18
AA (μM)	0.77 ± 0.12	0.54 ± 0.31	0.08
Count (1000/mm^3)	257 ± 21	274 ± 35	0.12
Control group ($n = 4$)			
ADP (μM)	2.85 ± 1.23	1.60 ± 0.90	0.10
Collagen (μg/mL)	0.58 ± 0.23	0.40 ± 0.06	0.17
AA (μM)	0.58 ± 0.21	0.65 ± 0.35	0.32
Count (1000/mm^3)	311 ± 40	315 ± 46	0.62

[a]Values reported as mean ± standard deviation. Abbreviation: AA, arachidonic acid; for other abbreviation see Table 1.

TABLE 6
Blood Coagulation Properties

	Study day[a]		
	30 Low-DHA diet	120 High-DHA diet	*P*-value
Intervention group ($n = 6$)			
Prothrombin time (s)	12.80 ± 0.27	12.93 ± 0.50	0.24
Activated partial thromboplastin time (s)	34.95 ± 4.38	33.67 ± 3.17	0.06
Antithrombin-III (%)	93.56 ± 3.20	89.81 ± 6.28	0.11
Control group ($n = 4$)			
Prothrombin time (s)	12.48 ± 0.36	12.38 ± 0.36	0.09
Activated partial thromboplastin time (s)	30.55 ± 4.38	29.25 ± 3.73	0.13
Antithrombin-III (%)	94.00 ± 6.02	98.97 ± 2.18	0.13

[a]Values reported as mean ± standard deviation. For abbreviation see Table 1.

counts when the results from the two diets are compared. Table 6 lists some values for the soluble blood coagulation parameters for these subjects before and after they consumed the high-DHA diet. Neither the PT nor APTT values were different after the subjects had consumed either diet, although the antithrombin-III was slightly lower after they had consumed the DHA diet; the difference did not reach statistical significance.

Figure 2 compares the total fatty acid composition of the whole platelets after the subjects consumed the high-DHA diet for 90 d with the composition after 30 d on the low-DHA. The DHA, linoleic acid, oleic acid, arachidonic acid, EPA, and docosapentaenoic acid content of the platelets showed statistically significant changes ($P < 0.05$) between the two diets. DHA appeared to replace arachidonic acid, with some reduction of oleic and linoleic acids also. EPA increased markedly in the volunteers' platelets despite the fact that they consumed none of this compound. Presumably the EPA was produced by retroconversion of DHA. This is normalized weight percentage data, and if the percentage of one or more fatty acids increases then, obviously, one or more fatty acids must decrease. The four control subjects did not show any significant changes between days 30 and 120 while they were consuming the low-DHA diet.

DISCUSSION

Epidemiological studies (32–34) have consistently found an inverse relationship between intake of fish or marine food and cardiovascular disease. Most investigators have made the assumption that it is the presence of n-3 polyunsaturated fatty acids in marine organisms that provides the beneficial effects (4–10). Furthermore, feeding studies with marine oils or pu-

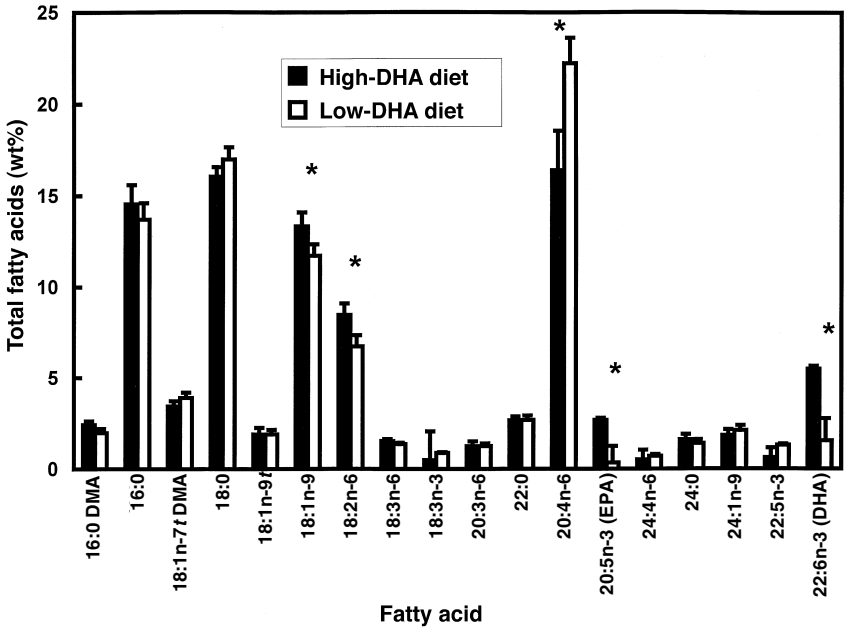

FIG. 2. The total fatty acid composition of the platelets after the consumption of the low-DHA (docosahexaenoic acid) and high-DHA diets by the intervention group in this study. The control group is not shown, but there were no differences among the various fatty acids compared after 30 d and 120 d on the stabilization diet. Individual fatty acids are designated by carbon chain length, number of double bonds in the molecule, and the position of the first double bond from the methylene end of the molecule. The asterisks designate fatty acids that are significantly different between the two diet periods with *P* values of less than 0.05. The vertical lines define one standard deviation from the means for the six subjects in the intervention group. Only fatty acids present in amounts greater than 0.5% of the total fatty acids are displayed in this figure. Abbreviations: DMA, dimethyl acetal; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

rified n-3 fatty acids tend to support this assumption (12, 14–16). Marine oils, containing various proportions of DHA and EPA when added to human diets, tend to reduce platelet aggregation and increase bleeding times (10–12,35). In this study, DHA in the absence of EPA was fed to human volunteers. Dietary DHA did not have significant effects on the major hemostatic parameters such as bleeding time or platelet aggregation, at least, not when consumed at 6 g/d for 90 d. Also, the soluble clotting factors as measured by the PT, the APTT, and antithrombin-III (considered a measure of the *in vivo* thrombotic tendency) were unchanged in the volunteers who consumed 6 g/d of DHA.

These findings are contrary to the report of von Schacky and Weber (16), who found that DHA caused a significant reduction in platelet aggregation in six subjects who consumed ethyl docosahexaenoate for 6 d. The retroconversion of DHA to EPA as reported by von Schacky and Weber (16) and others (3,36) was also observed. Perhaps there is a compensatory adaptation to the higher levels of DHA in the circulation when DHA is fed for a longer period. Thus, von Shacky and Weber (16) may have measured a transitory change in platelet function that the experimental design in our study would not detect. Also, Sanders and Hinds (37) observed an increase in the lag time for collagen-induced platelet aggregation when fish oil was consumed. No similar lag time was found in this study when DHA was the only n-3 fatty acid in the diet.

It should be noted, however, that EPA appears in the blood and tissues in levels equivalent to that obtained when EPA is fed to subjects in marine oils or seafoods (16,18,36) when DHA is fed in the absence of EPA. Conquer and Holub (36) recently demonstrated that dietary DHA, free of EPA, was retroconverted to EPA at a rate of 9.4%. In an accompanying paper (38) we report that dietary DHA does lower plasma triglyceride levels similarly to fish oils and purified EPA preparation. As the plasma EPA levels in these volunteers rose significantly, EPA could still be the physiologically active agent that caused the triglyceride lowering.

Nevertheless, dietary DHA in this study did not cause the changes in hemostatic physiological parameters attributed to fish oils or EPA. Indeed, even at 6 g/d, a level that is about 100 times the typical intake of DHA in the United States (39), it appears to have no short-term adverse consequences. In another aspect of this reported elsewhere (40), it was observed that dietary DHA does inhibit the production of thromboxane, but this inhibition is less than that observed when EPA is fed to humans. Furthermore, dietary DHA does not suppress prostacyclin production while dietary EPA does (40).

The retroconversion of DHA to EPA observed in this work and in studies by other investigators (16,18,36) raises interesting questions regarding these findings that dietary DHA has little effect on the hemostatic parameter in normal human males. The levels of EPA found in the circulation of the volunteers in this study are equivalent to the levels reported by other investigators (37) in studies in which significant changes in several hemostatic parameters, such as bleeding times and *in vitro* platelet function, were detected. In an earlier study from this laboratory, feeding salmon for 50 d caused a slight change in platelet aggregation in response to ADP. Of course, salmon does contain both EPA and DHA, although more DHA than EPA. These paradoxical findings are difficult to explain, as is the finding of von Schacky and Weber (16), that DHA was more effective than EPA in reducing platelet aggregation. EPA synthesized by retroconversion may be compartmentalized and unable to interact with appropriate trigger mechanisms. This is possible but not likely. Another explanation is that our experiment was too short to detect the effects described by others, although the feeding period in this study was longer than many of the studies in the literature (16,37,41) but less than others (42–44). Also, it should be noted that there are a number of reports (45–47) of studies that did not find changes in hemostatic function after fish oil supplementation. However, as platelet aggregation tests are notoriously variable, and the number of subjects in this study was small, it may be that the power of the study was too small to detect changes, although this is unlikely because of the high dosage, 6 g/d of DHA, fed in this study.

Von Schacky and Weber (16) did not find any changes in the platelet fatty acid composition after supplementation with DHA for 6 d. In contrast Conquer and Holub (36), after feeding 1.6 g/d of DHA for 42 d, reported marked changes in the platelet total fatty acids quite similar to those reported here. Apparently little exchange of fatty acids between platelets and plasma occurs *in vivo*. Thus, changes in platelet fatty acid composition caused by changes in dietary fatty acid intake are probably produced by incorporation of fatty acids into the megakaryocytes in the bone marrow during the platelet formation and maturation process. In this study even more DHA was incorporated into the volunteers' platelets than observed by Conquer and Holub (36) presumably owing to the higher dosage fed to the volunteers.

It is difficult to correlate changes in platelet fatty acid composition with platelet functions or *in vitro* aggregation. In an earlier study done here (35), the consumption of a salmon diet caused a modest change in the EPA and DHA levels in the volunteers' platelets, and these changes for both EPA and DHA were less than those found when 6 g/d of DHA were fed. Yet the salmon diet caused a small decrease in platelet aggregation by ADP, a phenomenon not detected here. A complete explanation of these observations must await further studies, feeding purified DHA or EPA and combinations of both.

The findings reported here and elsewhere (5,7–9,15,36) suggest that in short-term human feeding studies, there are few adverse effects from markedly increased levels of DHA in the diet. Tracer studies (48,49) show that very little α-linolenic acid is converted to DHA in human adults; thus, dietary DHA may be helpful in replacing tissue stores of DHA without the need to convert precursor fatty acids to DHA. However, it would be prudent to increase the daily intake of antioxidant vitamins if significant quantities of highly unsaturated fatty acids are added to the diet in light of the current concern over oxidized low-density lipoproteins (50,51), free

radical damage to cells (52,53), and the free-radical theory of aging (54).

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The Effect of Dietary Docosahexaenoic Acid on Plasma Lipoproteins and Tissue Fatty Acid Composition in Humans

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ABSTRACT: Normal, healthy male volunteers ($n = 6$) were fed diets [high docosahexaenoic acid-DHA] containing 6 g/d of DHA for 90 d. The stabilization (low-DHA) diet contained less than 50 mg/d of DHA. A control group ($n = 4$) remained on the low-DHA diet for the duration of the study (120 d). Blood samples were drawn on study days 30 (end of the stabilization period), 75 (midpoint of the intervention period), and 120 (end of the intervention period). Adipose tissue (AT) samples were taken on days 30 and 120. The plasma cholesterol (C), low density lipoprotein (LDL)-C and apolipoproteins (apo) [AI, B, and lipoprotein (a)] were unchanged after 90 d, but the triglycerides (TAG) were reduced from a mean value of 76.67 ± 24.32 to 63.83 ± 16.99 mg/dL ($n = 6$, $P < 0.007$ using a paired *t*-test) and the high density lipoprotein (HDL)-C increased from 34.83 ± 4.38 mg/dL to 37.83 ± 3.32 mg/dL ($n = 6$, $P < 0.017$ using a paired *t*-test). The control group showed no significant reduction in plasma TAG levels. Apo-E, however, showed a marked increase in the volunteers' plasma after 90 d on the high-DHA diet, from 7.06 ± 4.47 mg/dL on study day 30 to 12.01 ± 4.96 mg/dL on study day 120 ($P < 0.002$ using a paired *t*-test). The control subjects showed no significant change in the apo-E in their plasma (8.46 ± 2.90 on day 30 vs. 8.59 ± 2.97 on day 120). The weight percentage of plasma DHA rose from 1.83 ± 0.22 to 8.12 ± 0.76 after 90 d on the high-DHA diet. Although these volunteers were eating a diet free of eicosapentaenoic acid (EPA), plasma EPA levels rose from 0.38 ± 0.05 to 3.39 ± 0.52 (wt%) after consuming the high-DHA diet. The fatty acid composition of plasma lipid fractions—cholesterol esters, TAG, and phospholipid—showed marked similarity in the enrichment of DHA, about 10%, after the subjects consumed the high-DHA diet. The DHA content of these plasma lipid fractions varied from less than 1% (TAG) to 3.5% (phospholipids) at baseline, study day 30. EPA also increased in all plasma lipid fractions after the subjects consumed the high-DHA diet. There were no changes in the plasma DHA or EPA levels in the control group. Consumption of DHA also caused an increase in AT levels of DHA, from 0.10 ± 0.02 to 0.31 ± 0.07 (wt%) ($n = 6$, $P < 0.001$ using a paired *t*-test), but the amount of EPA in their AT did not change. Thus, dietary DHA will lower plasma TAG without EPA, and DHA is retroconverted to EPA in significant amounts. Dietary DHA appears to enhance apo-E synthesis in the liver. It appears that DHA can be a safe and perhaps beneficial supplement to human diets.

Lipids 32, 1137–1146 (1997).

Dietary marine oils may have beneficial roles in the prevention of cardiovascular disease (1–3) and other diseases (4). These oils contain a high content of long-chain n-3 polyunsaturated fatty acids, mainly eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Fish oils and purified EPA are powerful antihypertriglyceridemic agents (5,6) and may now be the agents of choice in the treatment of uncomplicated hypertriglyceridemia (7). However, it is not known if dietary DHA without EPA has the same antihypertriglyceridemic properties as EPA or fish oil. Although there are many studies in the scientific literature in which fish (8–11), fish oil supplements (12–16), or purified EPA (16–18) have been fed to human volunteers, there are only a few reports in which investigators have fed only DHA without EPA (19,20).

DHA is a normal constituent of the human diet (21) if one is not a vegan, and is found in most cells and tissues in significant amounts (22). DHA appears to have a major role in human physiology, particular in the development of the nervous system and in normal vision (23). *In vivo* DHA can be retroconverted to EPA (19,24). Recently, considerable interest in the metabolism and physiological function of dietary DHA has developed (25–27). Also, as plasma lipids and lipoprotein (Lp), and perhaps the circulating fatty acids associated with plasma lipids, are predictors of cardiovascular risk, it seems important to learn if dietary DHA has independent effects on blood lipids and lipid metabolism in general. In view of the lack of controlled human-feeding studies and the unresolved question about the physiological effects of dietary DHA coupled with the commercial availability of a natural triglyceride (TAG) containing about 40% DHA, we conducted a human-feeding study with DHA in a metabolic research unit (MRU). Normal human volunteers were chosen

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Abbreviations: AA, Arachidonic acid; apo, apolipoprotein; AT, adipose tissue; C, cholesterol; DHA, docosahexaenoic acid; DPA, docosapentaenoic; EPA, eicosapentaenoic acid; FA, fatty acid; FFA, free fatty acids; HDL, high density lipoprotein; LDL, low density lipoprotein; Lp, lipoprotein; MRU, metabolic research unit; RBC, red blood cells; TAG, triglycerides.

to avoid confounding by lipid abnormalities and to obtain baseline data. The dose of DHA was based on the maximum amount consumed by volunteers in earlier studies. The detailed protocol and design of the study have been described in a previous paper (28).

This paper reports the effects on several lipid parameters of supplementing a natural food diet (containing <50 mg/d DHA) with 6 g/d of DHA as a TAG. The supplementation period was 90 d. Results from analyses of blood lipid levels, fatty acid (FA) composition of the plasma, red cells and adipose tissue (AT), Lp cholesterol levels, and selected apolipoprotein (apo) levels are presented.

STUDY DESIGN

The design was a single-blind, feeding study in which 12 healthy volunteers lived in the MRU of the Western Human Nutrition Research Center for 120 d. All subjects were fed a stabilization diet (low-DHA), about 30% of calories from fat, for 30 d after they entered the MRU. On day 31 of the study, eight of the subjects, designated Group A, were switched to a diet containing 6 g/d of DHA (from 15 g of DHASCO® oil; Martek Biosciences Corporation, Columbia, MD). The remaining four subjects, designated Group B, continued to consume the low-DHA diet that contained less than 50 mg of DHA. The subjects were discharged from the MRU on day 120 of the study. For further details see the previous paper (28).

Subjects. The physical characteristics of the subjects and the recruitment process were described in the previous paper (28). The Institutional Review Boards of the University of California at Davis and the U.S. Department of Agriculture approved the protocol for this study. Initially, 12 volunteers were included in the study, but two subjects, both from group A, were unable to complete the protocol.

During confinement, blood was drawn on study days 2, 30, 75, and 120. AT samples were taken on study days 30 and 120. Although we analyzed the various parameters under study on all volunteers on these study days, values from samples obtained on days 30 and 120 were used for statistical analysis.

MATERIALS AND METHODS

A description of the oil (DHASCO®) and its FA composition is given in the previous paper (28). The oil was kept under nitrogen and at −20°C until it was needed for the food preparation. Open bottles were flushed with N_2 before they were resealed and returned to the freezer. As the DHA was fed in a cold yogurt cocktail, it is unlikely that any oxidative degradation took place between the time the food was prepared and when volunteers ate it.

Diets. The nutrient composition of the diets was calculated from a computerized nutrient data bank using the data from *USDA Handbook* (29) and adjusted to provide at least the RDA for known essential nutrients (30). A detailed description of the diets is given in the previous paper (28). A complete description of the diets, listing all the major and minor nutrients, is available upon request.

Measurements. Blood was drawn according to the schedule described above between 7:00 and 8:00 A.M. after an overnight fast. It was drawn into vacutainers with appropriate anticoagulants (either citric acid, 1% solution, or EDTA depending on the subsequent use of the sample) using a Teflon catheter (Angiocath; Deseret Medical, Sandy, UT) for FA analysis. Red cells for FA analysis were prepared from the residue by resuspending the cells in 10 mL of phosphate buffered saline and recentrifuging the cells at $400 \times g$ for 15 min three times. The supernate was discarded. The red cells were frozen at −70°C until extraction of the lipids. AT biopsy samples were taken from the buttocks using a hypodermic syringe equipped with 13 gauge stainless-steel needle (28). The samples (10 to 50 mg) were immediately transferred to cold (0°C) isotonic saline, then placed in a −70°C freezer within an hour and stored until further processing.

AT FA analyses. The FA analyses of the AT samples were done by procedures published previously (31). Briefly, the tissue was extracted with $CHCl_3$/MeOH (2:1, vol/vol), the extract transmethylated with methanolic HCl (7%), the methyl ester extracted with hexane, and the FA analyzed by gas–liquid chromatography as mentioned below.

Lipid extraction and FA analysis. Plasma and red cell samples were extracted by the procedures described by Nelson (32,33) using chloroform/methanol (2:1, vol/vol). Samples were then transmethylated with methanolic HCl (7%, w/w) by the procedures described previously (34,35). When the total lipid extract was transmethylated, the impurities, extracted into the hexane phase after termination of the reaction, were removed by thin-layer chromatography as described elsewhere (33). The conditions of the capillary GLC have been described previously (34). Briefly, the column was a 30-m × 0.025-mm fused quartz column coated with SP-2340 (Supelco, Inc., Bellefonte, PA). The GLC data were processed with a Hewlett-Packard ChemStation software (Palo Alto, CA) running on an IBM compatible desktop computer.

Apo analysis. The apo AI (36) and B (37) were analyzed using commercial kits (Sigma Diagnostics, St. Louis, MO). The apo E (38) and Lp(a) (39) were determined using commercial ELISA kits (Perlimmune, Inc., Rockville, MD).

Plasma lipid analyses: triglyceride (TAG) and cholesterol determinations. Plasma cholesterol, TAG, high density lipoprotein (HDL)-cholesterol, and low density lipoprotein (LDL)-cholesterol were analyzed in a Cobas-Faras centrifugal analyzer (Hoffman-La Roche, Nutley, NJ) using automated enzymatic methods as described by McGowan *et al.* (40) and Allain *et al.* (41).

Statistical analysis. The lipid, Lp, and apo data were analyzed using the paired *t*-test with $n = 6$ for the intervention group (A) or $n = 4$ for the control group (B) or analysis of variance with repeated measures. The subjects acted as their own controls; statistically significant differences were determined by comparing values of the various parameters on study days 30 (baseline) and 120 (end of study). No statisti-

cal comparisons were made between the control group and the intervention group due to the small size of the groups. The control group was included to detect temporal drift while the volunteers were confined to the MRU. The fatty acid data were analyzed with a single tailed *t*-test. All the statistical analyses were performed with the PC SAS data system, version 6.11, obtained under license from the SAS Institute, PC-SAS, (*SAS PC Manual*, SAS Institute, Chapel Hill, NC, 1993).

RESULTS

Blood lipids. The blood lipid values of the volunteers on entry to the study for both the control and intervention groups are given in Table 1. Table 2 lists the blood lipid values at study days 30 (baseline) and 120 (end of intervention) for both groups. The addition of 6 g/d of DHA to a natural-food diet for 90 d did not affect the total plasma cholesterol value or the LDL-cholesterol (C) value in these volunteers (See Table 2); however, the plasma TAG levels decreased significantly ($P < 0.004$, using a paired *t*-test). The HDL-C value rose 3 mg/dL and was significant at $P < 0.02$. The control group's TAG, LDL-C, and HDL-C values were unchanged after the group had consumed the low-DHA diet for 90 d. There was a slight decrease in the control group's plasma total cholesterol value, $P < 0.03$, that can be attributed to the low-cholesterol, low-fat diet given to them during this study.

TABLE 1
Blood Lipid Values of Subjects on Entry into the Docosahexaenoic Acid Feeding Study[a]

	Control group[b] (mg/dl)	Intervention group[c] (mg/dl)
Total cholesterol	188.0 ± 60.1	186.8 ± 25.3
LDL-cholesterol	121.7 ± 32.9	129.2 ± 17.2
HDL-cholesterol	46.3 ± 9.4	37.7 ± 8.1
Triacylglycerols	102.3 ± 83.6	99.5 ± 40.1

[a]Mean values ± SD.
[b]$n = 4$.
[c]$n = 6$; LDL, low density lipoprotein; HDL, high density lipoprotein.

Apoproteins. The plasma values for apo-A1, apo-B, and Lp(a) showed very little change (see Table 3) before and after the volunteers consumed the high-DHA diet. Although apo-A1 level decreased about 7 mg/dL and the Lp(a) decreased about 4 mg/dL, these changes were not statistically significant (Table 2); however, the Lp(a) levels rose significantly in the control group ($P < 0.01$), perhaps attributable to the low cholesterol, low-fat diet given the volunteers, although this may have been a statistical quirk due to the small number of volunteers in the control group. Apo-E, however, showed a marked increase in its plasma concentration after the subject had consumed the high-DHA diet, from 7.06 ± 4.47 mg/dL on study day 30 to 12.01 ± 4.96 mg/dL on study day 120 ($P < 0.002$ using a paired *t*-test). The control subject showed no significant change in their apo-E content in their plasma (8.46 ± 2.90 on day 30 vs. 8.59 ± 2.97 on day 120).

Plasma fatty acids. Significant changes in the total plasma FA composition in all subjects in the intervention group were observed, with a fourfold increase in the total plasma DHA level and a similar increase in the plasma EPA level after the consumption of the high-DHA diet by the volunteers. (Fig. 1). In the control group there were no significant changes in the FA composition of the subjects' tissues during the 90 d when they consumed the low-DHA diet (data not shown). In the intervention group, a reduction in the plasma oleic acid, linoleic and arachidonic acid (AA) contents was noted. None of the other plasma FA levels changed significantly. The changes in the FA composition of the individual lipid classes showed few differences from the pattern found in the total plasma FA composition. On study day 30 (low-DHA diet) the volunteers' plasma TAG contained little DHA, about 0.6%. This increased to almost 10% on the high-DHA diet (Fig. 2). Also, the EPA levels went from less than 0.3 to 1.7% in these volunteers after 90 d on the high-DHA diet. Most of the increases were compensated by a decrease in the oleic and linoleic acid contents of the TAG. The changes in the DHA and EPA levels in the cholesterol esters (Fig. 3) were similar to those observed in TAG, although the main reduction was in the palmitic acid content while the oleic and linoleic acid contents were largely unchanged. No other FA showed sig-

TABLE 2
Effect of Docosahexaenoic Acid (DHA) Supplementation on Plasma Cholesterol, Lipoprotein Cholesterol, and Triglyceride Concentrations[a]

	Intervention group[b] (mg/dl)		Control group[c] (mg/dl)	
	Low-DHA Diet (study day 30)	High-DHA Diet (study day 120)	Low-DHA Diet (study day 30)	Low-DHA Diet (study day 120)
Triglycerides	72.5 ± 17.8	53.8 ± 15.5	109.0 ± 97.5	95.8 ± 77.6
P-value (SD 30 vs. SD 120)		0.004		0.174
Total cholesterol	163.8 ± 17.9	161.7 ± 25.3	188.8 ± 55.4	174.3 ± 58.5
P-value (SD 30 vs. SD 120)		0.303		0.034
LDL-cholesterol	114.5 ± 17.3	113.0 ± 24.0	128.0 ± 45.9	126.9 ± 34.6
P-value (SD 30 vs. SD 120)		0.256		0.456
HDL-cholesterol	34.8 ± 4.4	37.8 ± 3.3	38.8 ± 5.6	35.5 ± 4.9
P-value (SD 30 vs. SD 120)		0.017		0.189

[a]Mean values ± $n = 6$. See Table 1 for other abbreviations.
[b]$n = 6$.
[c]$n = 4$.

TABLE 3
Apoprotein (Apo) Levels in Plasma of Subjects After the High-DHA or Low-DHA Diets[a]

	Intervention group[b] Apo (mg/dL)		Control group[c] Apo (mg/dL)	
	Low-DHA Diet (study day 30)	High-DHA Diet (study day 120)	Low-DHA Diet (study day 30)	Low-DHA Diet (study day 120)
Apo-A-1	103.2 ± 3.8	96.4 ± 8.3	112.9 ± 12.3	103.7 ± 10.8
P-value (SD 30 vs. SD 120)		0.06		0.34
Apo-B	47.3 ± 6.5	45.3 ± 73.2	57.2 ± 21.8	51.5 ± 22.3
P-value (SD 30 vs. SD 120)		0.38		0.52
Apo-E	7.1 ± 4.5	12.0 ± 5.0	9.9 ± 2.9	8.6 ± 3.0
P-value (SD 30 vs. SD 120)		0.002		0.85
Lp(a)	50.2 ± 87.8	46.3 ± 82.8	27.9 ± 19.6	32.1 ± 17.2
P-value (SD 30 vs. SD 120)		0.22		0.01

[a]Mean values ± SD. Lp, lipoprotein; see Table 2 for other abbreviation.
[b]$n = 6$.
[c]$n = 4$.

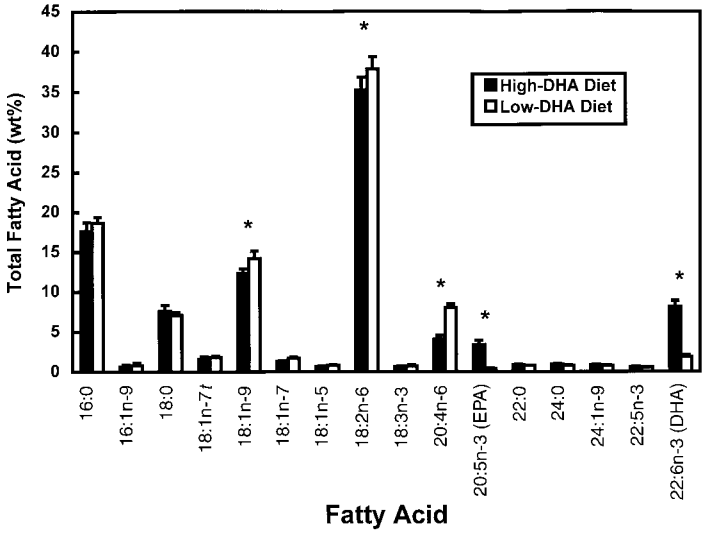

FIG. 1. The mean ($n = 6$) values for the major fatty acids in the subjects' plasma after the subjects in the intervention group had been on the low-docosahexaenoic acid (DHA) (30 d) or high-DHA diet (90 d). The asterisks indicate fatty acid values that differed significantly by at least $P < 0.05$ between the two dietary periods. The vertical lines define one standard deviation from the means for the six subjects in this study. Fatty acids are designated by chain length, number of double bonds in the molecule, and the position of the first double bond from the methylene end of the molecule. A "*t*" at the end of the notation is an abbreviation for a *trans* fatty acid. EPA, eicosapentaenoic acid.

nificant changes in the cholesterol ester fraction. The phospholipid fraction's FA composition underwent similar changes with a marked increase in DHA and EPA contents after the subjects consumed the high-DHA diet (Fig. 4). The DHA level rose from 3.1 to 10.1%, and the EPA content rose from 0.6 to 3.8%. In the phospholipid fraction, however, the AA content dropped noticeably, from 9.2 to 5.6 %, while most of the other major FA showed little change.

Adipose tissue. The total FA composition of the AT is shown in Figure 5. Table 4 lists the DHA and EPA content in the AT. The volunteers' AT contained very little DHA (<0.15%) before consumption of the high-DHA diet; the DHA content of the AT underwent a threefold increase after the volunteers consumed the high-DHA diet for 90 d. The EPA level in the AT did not change significantly during the feeding period, although it should be noted that it was approximately five times the DHA level at baseline.

Red blood cells (RBC). Figure 6 shows the FA composition of the RBC before and after dietary supplementation with DHA. DHA supplementation appeared to increase the level of palmitic, stearic, and DHA and lower the level of linoleic acid and AA in the red cell.

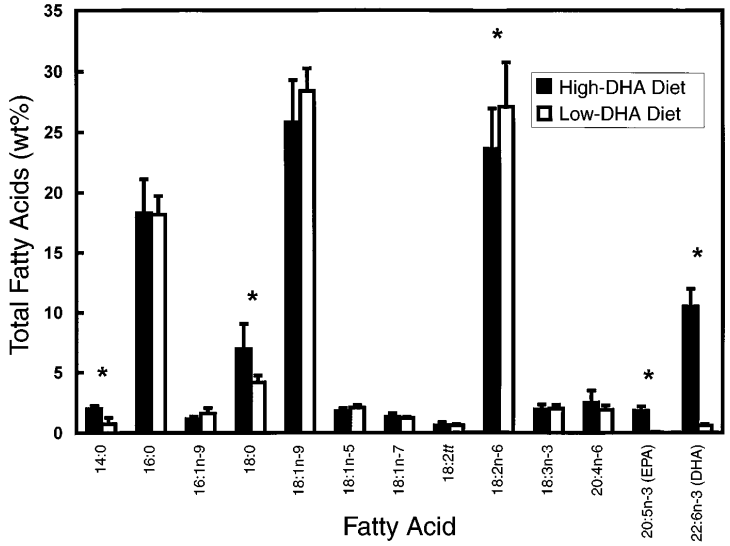

FIG. 2. The mean (n = 6) values for the major fatty acids in the subjects' plasma triglyceride fraction after the subjects in the intervention group had been on the low-DHA (30 d) or high-DHA diet (90 d). The asterisks indicate fatty acid values that differed significantly by at least $P < 0.05$ between the two dietary periods. The vertical lines define one standard deviation from the means for the six subjects. Fatty acid abbreviations are described in the caption to Figure 1.

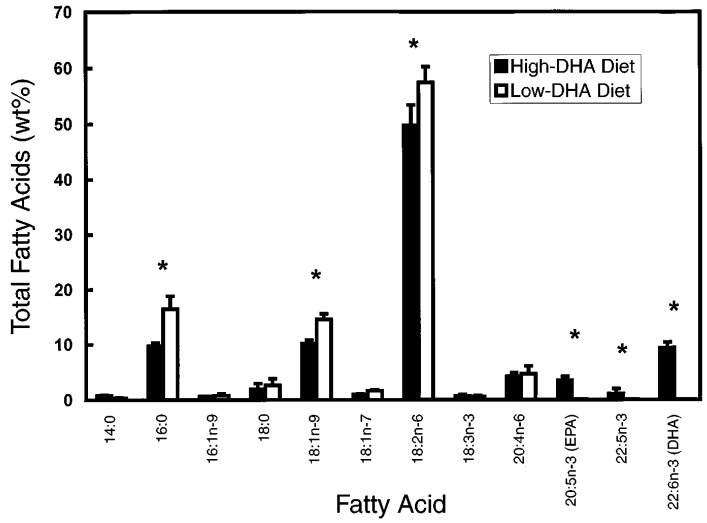

FIG. 3. The mean (n = 6) values for the major fatty acids in the subjects' plasma cholesterol ester fraction after the subjects in the intervention group had been on the low-DHA (30 d) or high-DHA diet (90 d). The asterisks indicate fatty acid values that differed significantly by at least $P < 0.05$ between the two dietary periods. The vertical lines define one standard deviation from the means for the six subjects. Fatty acid abbreviations are described in the caption to Figure 1.

DISCUSSION

When the DHA in the diet was increased for 90 d from less than 50 mg/d to 6 g/d in an isocaloric substitution for linoleic acid, there was a marked decrease in the plasma TAG level without a corresponding change in the plasma cholesterol level. In this diet, about 2% of calories were polyunsaturated n-3 fatty acids (DHA) while the linoleic acid content of the diet was reduced by an equal amount while holding the saturated and monounsaturated fat content of the diet constant. The Lp patterns did show a reduction in the very low density lipoprotein and a rise in the HDL fractions without a change in the LDL fraction. The individuals studied in this work were all healthy, normolipidemic subjects (28), and achieving lipid

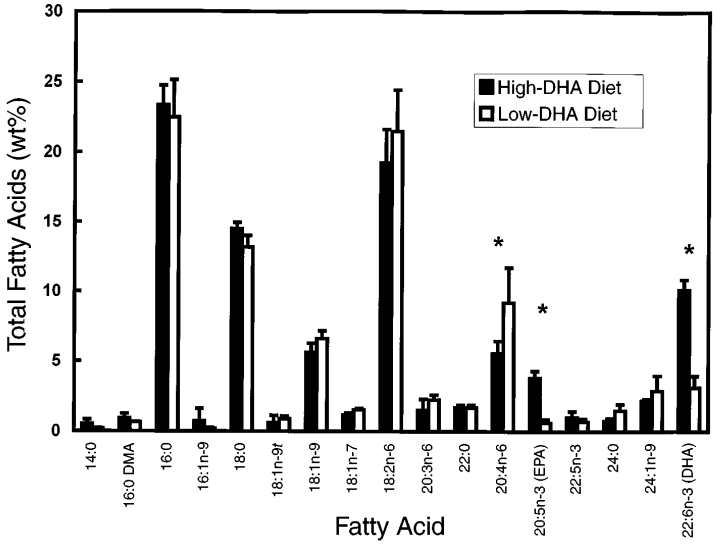

FIG. 4. The mean (n = 6) values for the major fatty acids in the subjects' plasma phospholipid fraction after the subjects in the intervention group had been on the low-DHA (30 d) or high-DHA diet (90 d). The asterisks indicate fatty acid values that differed significantly by at least $P <$ 0.05 between the two dietary periods. The vertical lines define one standard deviation from the means for the six subjects. Fatty acid abbreviations are described in the caption to Figure 1.

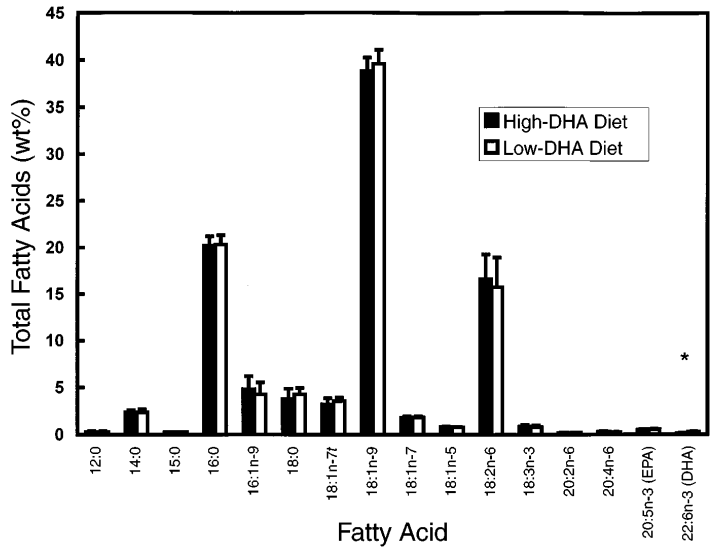

FIG. 5. The mean values for the major fatty acids in the subjects' adipose tissue after the subjects in the intervention group had been on the low-DHA (30 d) or high-DHA diet (90 d). The asterisks indicate fatty acid values that differed significantly by at least $P < 0.05$ between the two dietary periods. The vertical lines define one standard deviation from the means for the six subjects. Fatty acid abbreviations are described in the caption to Figure 1.

reductions in normolipidemic persons is more difficult than in hyperlipemic subjects (42,43). Thus, increasing the DHA content of the diet in hypertriglyceridemic individuals may cause a more marked reduction in plasma TAG levels than observed here. Also, the polyunsaturated/saturated ratio was constant in this study at about 1:1. Hayes and Khosla (44,45) have suggested that plasma lipid levels show little alteration with changes in the amount of FA in the diet if the linoleic acid content of the diet is above 7%, as it was in this study.

Perhaps the most distinctive change in the plasma Lp observed after DHA feeding was the marked increase in the apo-E content. To our knowledge, this increase in plasma apo-E content after feeding n-3 FA to people has not been reported previously. Although several studies (46–48) have shown that feeding of n-3 FA, particularly EPA, decreased apo-B secretion from the liver, though not necessarily a decrease in plasma apo-B concentrations (49), apo-E was usually not measured in these studies (46–49). Other investigators have

TABLE 4
DHA and Eicosapentaenoic Acid Contents of Adipose Tissue After High-DHA or Low-DHA Diets[a]

Group	Fatty acid	Study day 30 (mean values ± SD)	Study day 120 (mean values ± SD)	*P*-value
Intervention	20:5n-3	0.48 ± 0.06	0.55 ± 0.10	0.130
Control	20:5n-3	0.42 ± 0.02	0.45 ± 0.07	0.303
Intervention	22:6n-3	0.10 ± 0.02	0.31 ± 0.07	0.001
Control	22:6n-3	0.07 ± 0.16	0.07 ± 0.68	0.412

[a]Total fatty acids (wt%).

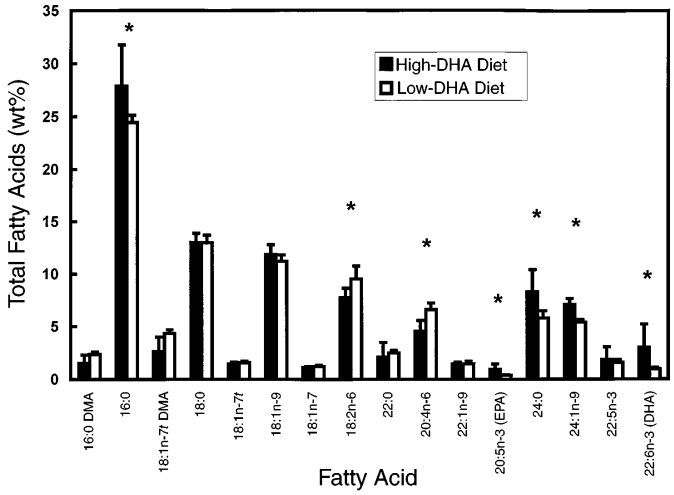

FIG. 6. The mean (n = 6) values for the major fatty acids in the subjects' red blood cells after the subjects in the intervention group had been on the low-DHA (30 d) or high-DHA diet (90 d). The asterisks indicate fatty acid values that differed significantly by at least $P < 0.05$ between the two dietary periods. The vertical lines define one standard deviation from the means for the six subjects. Fatty acid abbreviations are described in the caption to Figure 1.

reported a decrease in Lp(a) after n-3 FA feeding (50,51). In this study the Lp(a) levels did decrease slightly, but the decrease was not statistically significant perhaps because the number of volunteers was too few. The slight decrease in the total cholesterol and Lp(a) levels in the control group may have been caused by the low-fat, high n-6 diet fed to these volunteers although the number of individuals (4) in the control group was small with a commensurately greater chance of a false positive result.

A 6 g/d increase in dietary DHA has significant effects on the FA profile of the plasma. Normally, the plasma FA profile reflects the dietary FA profile (44,52,53). The observations reported from this study regarding the plasma FA composition of individuals consuming DHA without dietary EPA agree well with the recent report of Conquer and Holub (20) in which they reported their data as mol% rather than wt%. They fed volunteers 1.6 g/d of DHASCO® compared with 6 g/d used here; however, the increase in total plasma DHA content was quite similar, from 2.2 mol% at day zero to 7.6 mol% after 42 d on the supplement. Conquer and Holub (20) also found that the EPA levels in their subjects rose from 0.73 mol to 1.16 mol% after the volunteers consumed DHA for 42 d. In an earlier study von Schacky and Weber (19) reported that in volunteers fed 6 g/d of ethyl DHA for 6 d, plasma phospholipid DHA levels doubled. These authors also found a significant increase in the EPA content of the plasma phospholipids, presumably due to retroconversion of DHA in their volunteers. Thus, increased dietary DHA causes a rapid increase in plasma DHA, but it is apparent from the data of von Schacky and Weber (19) that retroconversion of DHA to EPA occurs within 6 d. In this study, no samples were taken in that period although there is no reason to assume that retroconversion was any slower here. Apparently, other body FA pools of DHA turn over much more slowly than the plasma FA pool (28).

In a typical, fasting normolipidemic individual, there is a pool of 15 g of plasma FA in the circulation based on a typical plasma total lipid level of 5 mg/dL and a 6-L blood volume. If one calculated the increase in the percentage of DHA found in the plasma in this study (from about 2 to 10%), much less than one day's dietary intake of DHA appears in the plasma (6 g/d fed vs. 2.5 g in the plasma pool). Even taking into account how much DHA is retroconverted to EPA and that found in the AT, only 3 to 3.5 g of the daily intake of DHA can be accounted for in the plasma of the volunteers

studied here. This observation suggests that an appreciable amount of DHA is retained in the liver and oxidized as rapidly as it is absorbed, which is consistent with observations on the disappearance of dietary α-linolenic and stearic acids (52,54). In contrast to this observation for n-3 FA, the situation with n-6 FA may be different. When 1.5 g/d of AA was fed to normal volunteers, most of it appeared in the plasma AA pool (55). Of course, the amount of AA fed in that study was only one-fourth the amount of DHA fed in this study.

The level of DHA in AT has been suggested as a convenient marker for habitual fish consumption by Marckmann *et al.* (56). In their report (56), the AT DHA levels, based on wt% of total FA, were almost identical to those reported here, 0.1% for 0 g/d of fish consumption to 0.3% for 70 g/d day of fish consumption. The EPA-to-DHA ratio in individuals who do not eat fish or fish products is about 5, and in fish-consuming individuals it drops to 1.6. Although the volunteers' plasma EPA content increased about 10 times, the AT EPA content in these volunteers did not change significantly during the study.

It should be noted, however, that the phospholipids were not separated from the TAG in the AT samples. FA from TAG make up about 98% of the total fatty acids in AT. Thus, the results given here represent primarily the TAG FA composition. As the phospholipids of AT have a different metabolic pathway from TAG, incorporation of DHA into the phospholipids of AT may have been different also although the data of Phinney and colleagues (31,57) and Nelson *et al.* (55) do not suggest that there are major differences between the incorporation of various FA in AT phospholipids and TAG.

Data from Phinney and colleagues (31,57) show that weight loss alone can alter the FA composition of AT. This suggests that release of fat from the AT during caloric deficiency is selective although there is no known mechanism to explain such a process. In individuals not consuming large amounts of DHA, its presence in AT is very low. Here a marked increase in the DHA level in the plasma TAG was observed while the increase in the level in DHA in the AT was much less. Of course, these subjects were in caloric balance, and their weight loss or gain was restricted to less than 2% of their body weight at entry into the study (28). Hence, little new fat was deposited in their AT. Apparently, only 400 mg of the total of 540 g of DHA consumed by the volunteers in this study was incorporated into their AT, based on the difference between the percentage of DHA in the AT at beginning and end of the intervention period. Thus, we can speculate that (i), the increased dietary DHA is rapidly metabolized to other compounds, hence not available for incorporation into AT, or (ii), that the turnover of FA in the AT is so slow in subjects with constant weight that a 90-d period is not long enough to equilibrate the AT. The data from Marckmann *et al.* (56) would suggest that this latter hypothesis is not the explanation as their subject did not have higher levels of DHA than those in this study even after 8 months on a high marine-oil diet. Hence, the former hypothesis is more likely.

The life span of RBCs is approximately 120 d in humans. These red cell FA composition data presented here represent a nonequilibrium state; hence, they are difficult to interpret. It is interesting, however, to note that the increase in the DHA level was small, but the decrease in the linoleic acid level in the red cells was much larger. The decrease in linoleic acid was compensated by increases in the palmitic and stearic acids levels rather than the DHA level in the red cells of these subjects. There is no readily apparent explanation for these changes. One can speculate, however, that increased dietary DHA interferes with incorporation of linoleic acid into RBC. The RBC then compensate by incorporating *in vivo* synthesized palmitic and stearic acids into their phospholipid in place of linoleic acid.

The intake of linoleic acid was reduced in the high-DHA diet by about 7 g/d compared to the low-DHA diet. It is possible that some of the effects observed in this study were due to the reduction of linoleic acid rather than the increase in DHA. This is unlikely, however, as the subjects were still receiving more than 20 g/d of linoleic acid on the high-DHA diet, an amount well above any known requirement for linoleic acid, and an amount that would flood the FA metabolic pathways. The low-DHA diet contained less than 50 mg of DHA per day and the high-DHA diet had 6 g/d. This is an increase of more than 120 times; certainly this dietary change is more likely to produce metabolic effects than a 20% reduction in the daily linoleic acid intake. Still, additional research is needed to resolve this issue definitely.

In conclusion, this study indicates that dietary DHA fed at 6 g/d lowers plasma TAG but does not affect cholesterol levels significantly. Dietary DHA at this level does markedly raise the DHA levels in the plasma. Also, dietary DHA is incorporated into AT but only in small amounts, about 0.1% of the total amount consumed during 90 d. The turnover of RBC is 120 d so equilibrium would not be obtained in a 90-d study, and changes in the FA composition of RBC may still occur when DHA is fed for longer periods. Still, the results from this study show that dietary DHA fed at 6.0 g/d for 90 d is well tolerated and does not cause any grastrointestinal side effects. In fact, dietary DHA may be beneficial in altering the individual's cardiovascular disease risk profile with respect to blood lipids and Lp that are risk factors for cardiovascular disease, but this remains to be established by longer-term studies.

Addendum. After this work was submitted, Vidgren *et al.* (58) published a report of a feeding study with human volunteers who consumed either fish (0.67 g/d DHA), fish oil (0.95 g/d DHA), or an algal oil rich in DHA (1.68 g/d) without any EPA. These authors reported the FA composition of the various plasma lipid fractions (TAG, cholesterol esters, and phospholipids), platelets, and RBC in a 14-wk, free-living study in which the diet was uncontrolled. Their results were similar to those reported here except that they reported increases in the DHA content in the various lipid fractions that were less than those found in this work presumably because the amount of DHA fed was about three times less per day (1.68 vs. 6.0 g/d) than the amount used in this study. The major difference between our results and those of Vidgren *et al.* (58) was that at 6 g/d of DHA incorporation of DHA into the plasma lipid

classes were about equal at 10% (wt%) of the total FA while Vidgren (56) found preferential incorporation DHA into the TAG and phospholipid fractions and little incorporation of DHA into cholesterol esters fraction. Thus, incorporation of dietary FA into particular plasma lipid fractions may be dependent on the amount in the diet.

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Pancreatic Bile Salt-Dependent Lipase Activity in Serum of Normolipidemic Patients

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ABSTRACT: Bile salt-dependent lipase (BSDL, E.C. 3.1.1.-) is a digestive enzyme secreted by the pancreatic acinar cell. Once in the duodenum, the enzyme, upon activation by primary bile salts, hydrolyzes dietary lipid esters such as cholesteryl esters and lipid-soluble vitamin esters. This enzyme is partially transferred from the duodenum or pancreas to the circulation where it has been postulated to exert a systemic action on atheroma-generating oxidized-low density lipoprotein (LDL). In the present study, sera from 40 healthy normolipidemic volunteers were used to investigate the possible linkage between circulating BSDL, lipids, and lipoproteins. We showed, firstly, that pancreatic-like BSDL activity can be detected in these serums. Secondly, BSDL activity increased significantly with the level of LDL-cholesterol and was also positively linked to the serum concentration of Apo B100 and Apo A-I. Thirdly, we also established that BSDL was associated with LDL, in part by a specific interaction with Apo B100, while no interaction was found with Apo A-I. No linkage with other recorded parameters (triglycerides, phospholipids, and high density lipoprotein-cholesterol) was detected. Because an increase in LDL-cholesterol represents an important risk factor for atheroma, the concomitant increase in BSDL, which can metabolize atherogenic LDL, suggests for the first time that this circulating enzyme may exert a positive effect against atherosclerosis.

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Pancreatic bile salt-dependent lipase (BSDL), also referred to as carboxyl ester lipase or bile salt-dependent cholesterol ester hydrolase, is a 100 kDa enzyme which hydrolyzes cholesteryl and lipid-soluble vitamin esters, triglycerides, and (lyso)phospholipids (1,2). Except for the latter substrates, bile salts are a prerequisite for hydrolysis (3). Although the enzyme was secreted into the duodenum where it exerted its digestive function, BSDL has been shown to transit throughout the enterocyte (4). As a consequence, BSDL is found in the blood plasma of humans (5) where lipoproteins are possible targets for the enzyme. Nevertheless, BSDL activity in serum cannot be measured on cholesteryl esters or triglycerides, owing partly to the competitive effect of circulating lipoproteins (6). It has effectively been shown that BSDL has the capability to hydrolyze low density lipoprotein (LDL)-cholesteryl esters in the presence of a concentration of bile salts achievable in plasma (7,8). Of physiological importance is the fact that BSDL reduces the atherogenicity of oxidized LDL by decreasing its lysophospholipid content (9). Evidence has accumulated to suggest that oxidized LDL is a key component in endothelial injury (10). Once formed by the endothelium, oxidized LDL may directly injure the vascular tissue and induce migration of monocytes and T lymphocytes in the intima (11). Oxidized LDL is enriched in lysophosphatidylcholine, which acts as a chemoattractant that recruits monocytes to the subintimal space (12). Once monocytes enter the subendothelial space, oxidized LDL may participate in the activation of monocytes into macrophages. These latter cells internalize LDL through scavenger receptors (13) and can oxidize LDL through several pathways (14). During this process, fatty acids undergo peroxidation which, in turn, yields a variety of reactive molecules, which can become covalently crosslinked to the apolipoprotein (Apo) B100 moiety of LDL particles. This could further exacerbate the atherogenic process. As a result, antioxidants such as probucol and vitamin E have been suggested as a means of preventing atherosclerotic lesions. Given the role attributed to lysophosphatidylcholine in the promotion of atherosclerosis, it has been proposed that BSDL could serve as a protective factor against the deleterious effects of oxidized LDL observed in atherosclerosis (9). Consequently, the aim of this study was twofold: (i) an attempt to accurately record BSDL activity in serum, and (ii) an investigation of whether the activity of circulating BSDL was linked to lipids and/or Apo present in serum.

EXPERIMENTAL PROCEDURES

Patients. Sera from 40 patients (27 women, 13 men, age range of 21–85 years) were used in this study. None had liver or pancreatic disease or was undergoing insulin treatment nor was taking corticosteroids, oral contraceptives or lipid-lowering agents. All were normoalbuminemic (>540 μmol/L). Blood (citrated) samples were drawn at 9:00 a.m. after an

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Abbreviations: Apo, Apolipoprotein; BSDL, bile salt-dependent lipase (E.C. 3.1.1.-); DFP, diisopropyl fluorophosphate; HDL, high density lipoproteins; LDL, low density lipoproteins; LPL, lipoprotein-lipase; VLDL, very low density lipoproteins.

overnight fast while subjects had rested for at least 20 min. The study protocol was approved by our Ethics Committee, and informed consent was obtained from all subjects.

Immunoprecipitation. Polyclonal antibodies (*pAb L64*) specific for human pancreatic BSDL were obtained, purified, and, when required, immobilized on agarose beads as described previously (15). Twenty µL of a suspension of immobilized *pAb L64* (corresponding to 100 µg of antibodies) was added (assays) to 100 µL of serum sample and incubated for 4 h at 25°C under agitation. Beads were then pelleted by centrifugation at 10,000 × *g* for 20 min. The esterolytic activity was recorded in the supernatant. Controls were performed under the same conditions, omitting immobilized antibodies. Under these conditions, the difference in activity between control and assay was representative of the BSDL activity in serum.

Enzyme assay. Esterolytic activity of serum samples was spectrophotometrically determined using 4-nitrophenyl hexanoate as substrate (16) at 404 nm in a thermostated cell at 30°C. The substrate was dissolved in 0.2 M Tris/HCl, pH 7.4 buffer containing NaCl (150 mM). Assays were performed in the absence or presence of sodium taurocholate (4 mM) as activator of BSDL. Under these conditions, the difference of measured activity was representative of the bile salt-activated esterolytic activity present in serum samples. Diisopropyl fluorophosphate (DFP)-inhibited esterolytic activity of serum was obtained after sample incubation (1 h at 25°C) in the presence of 4 mM DFP (stock solution 1 M in dry isopropyl alcohol) or of vector alone. Then the esterolytic activity of serum was recorded in the presence of bile salts as described before. The difference in activity before and after DFP treatment represents the DFP-inhibited esterolytic activity. Unless otherwise stated, DFP-inhibited esterolytic activity represents the activity of BSDL (see below).

Polyacrylamide gel electrophoresis (PAGE) and Western blotting. PAGE in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed according to Laemmli (17). Replicas of SDS-PAGE on nitrocellulose membranes were obtained after electrotransfer. These replicas were then treated as previously described (18) using *pAb L64* as the primary antibody. Alternatively, SDS-PAGE was stained with Coomassie blue R250 (Fluka, Buchs, Switzerland).

Apo preparation. Human plasma was adjusted to a density of 1.210 g/cm^3 with solid KBr then centrifuged in a SW 40 rotor (Beckman Instruments, Palo Alto, CA) at 100,000 × *g* for 24 h at 4°C. The supernatant containing mixed very low density lipoprotein (VLDL), LDL, and HDL was again centrifuged under the same conditions and finally dialyzed for 24 h against a 10 mM Tris/HCL buffer pH 7.4 (154 mM, NaCl; 1 mM EDTA; and 0.01% NaN_3). VLDL, LDL, and HDL were isolated by ultracentrifugation on a KBr density gradient according to Chapman *et al.* (19), after chylomicrons had been removed by preliminary centrifugation at 10,000 × *g* for 30 min at 4°C. Apo B100 and Apo A-I were then isolated, respectively, from LDL and HDL preparations as described by Karlin *et al*. (20). Their integrity was analyzed by SDS-PAGE and Western-blot using specific antibodies to Apo B100 and Apo A-I from Sigma Diagnostics (St. Louis, MO).

Apo immobilization. Human Apo B100 and Apo A-I were linked to Sepharose (Pharmacia, Uppsala, Sweden) using epoxy-activated Sepharose 6B (Pharmacia) according to the manufacturer's protocol. Under the conditions used, more than 80% of the Apo was coupled to Sepharose beads. Excess reactive groups, after immobilization of Apo B100 or Apo A-I (or all reactive groups of the control gel), were blocked with 1 M ethanolamine. In a typical experiment, 2.0 mg of pure Apo A-I (i.e., 70 nmoles) or 7.4 mg of Apo B100 (i.e., 15 nmoles) were coupled to 1 g of epoxy-activated Sepharose 6B gel. After exhaustive washings, gels were stored at 4°C in 0.1 M sodium bicarbonate buffer, pH 8.0 (0.5 M NaCl).

BSDL iodination. BSDL was purified from human pancreatic juice as previously described (3); then 10 µg of the pure protein was iodinated with ^{125}I-Na by the chloramine-T method. The radiolabeled BSDL (8 mCi/mg protein) was chromatographed on a Sepharose 6B column (1 × 60 cm), equilibrated, and eluted with 10 mM sodium phosphate buffer (pH 7.4) before use. During the iodination process, no BSDL degradation could have occurred because radiolabeled BSDL comigrates with the native BSDL on the Sepharose 6B column, and more than 87% of radiolabeled BSDL was precipitable with trichloroacetic acid. Stored at 4°C, this preparation remained stable for at least one month.

Binding of BSDL to lipoproteins. Isolated lipoproteins (500 µL; concentration adjusted to the initial plasma concentration) were incubated for 15 min at 37°C in the presence of 400 ng of ^{125}I-BSDL (4 pmoles, 600 ± 35 cpm/ng of BSDL) in 10 mM Tris/HCl buffer pH 7.4 (containing 154 mM NaCl, 1 mM EDTA, and 0.01% NaN_3). At the end of the incubation period, the mixture was chromatographed on a fast protein liquid chromatography-Superose 6 column (Pharmacia) (2 × 60 cm) eluted (0.5 mL/min) with the dialysis buffer. Radioactivity and optical density at 280 nm were monitored in each fraction. A blank experiment was performed by gel filtration under the same conditions of 400 ng of ^{125}I-BSDL added to 20 µg of pure BSDL (40 µg/mL) as a vector.

Interaction between BSDL and Apo. Sepharose-immobilized Apo beads were equilibrated and suspended in 10 mM Tris/HCl buffer pH 7.4 (1 mM EDTA; 0.15 M NaCl), adjusted to the same final amount of gel corresponding to 1 nmole of Sepharose-immobilized Apo B100 or to 5 nmoles of Sepharose-immobilized Apo A-I and pelleted. Immobilized-Apo were finally suspended in 1 mL of buffer and supplemented with approximately 1 pmole of ^{125}I-BSDL and with increasing amounts (from 0 up to 0.50 nmole) of unlabeled BSDL. Tubes were then incubated overnight at 4°C under gentle agitation. The tubes were quickly centrifuged on a benchtop centrifuge, gel pellets were washed several times with the incubation buffer, and the remaining radioactivity of the pellet suspended in counting liquid was recorded. Corrections for nonspecific binding were performed by using adequate amounts of control gel and treated under the same conditions.

Other procedures. Serum concentrations of phospholipids, triacylglycerol, cholesterol, and glucose were measured in duplicate by enzymatic methods (Boehringer Mannheim, Ger-

many; BioMérieux, Charbonnières-les-Bains, France; Roche Diagnostic Systems, Neuilly, France; and Sigma Diagnostics). Apo A-I and Apo B100 were evaluated by turbidimetric methods using specific antiserum to Apo (Turbiquant kit from Behring, Marbur, Germany). HDL-cholesterol was determined after precipitation of LDL and VLDL by phosphotungstic acid in the presence of magnesium ions (HDL-cholesterol kit from BioMérieux). The supernatant, obtained after centrifugation, contained the HDL from which the cholesterol was determined as above. LDL-cholesterol was assayed as described above after specific precipitation of LDL using polycyclic anionic surfactant (LDL-cholesterol kit from BioMérieux).

Statistical analysis. Results were expressed as means ± SD. The significance of differences was examined using Student's *t*-test. Regression lines were calculated by the least-squares method from individual data. Statistical comparisons between regression lines were performed by analysis of covariance. Probability values of $P < 0.01$ were considered significant. Because of serum volume needs to perform this study, not all determinations were done on each serum sample. Consequently, we provided the number of samples, *n*, used for statistical analysis along with the *P* value and the linear regression factor, *r*.

RESULTS

BSDL in serum. First we attempted to find a specific assay for BSDL activity in serum. For this purpose, the activity of BSDL present in serum of normolipidemic patients was estimated by an *in vitro* assay using 4-nitrophenyl hexanoate as substrate. Under our conditions, the difference in activity recorded in the presence or absence of bile salt represents the bile salt-activated esterolytic activity in serum. In another assay, serum samples were either mock-treated or treated with DFP (a potent inhibitor of BSDL) for 1 h at room temperature. At the end of incubation, the esterolytic activity was recorded. The difference in these activities represents the DFP-inhibited esterolytic activity of the serums. The bile salt-activated and DFP-inhibited esterolytic activities, determined on 10 different samples, gave similar results; 0.11 ± 0.05 and 0.12 ± 0.04 U/mL, respectively. These values, which correspond to 27 ± 6% of the total esterolytic activity present in serum, corroborate with the amount of esterolytic activity on 4-nitrophenyl hexanoate (0.11 ± 0.05 U/mL) which can be specifically precipitated by polyclonal antibodies directed against BSDL. The esterolytic activity of serum which was inhibited by DFP appeared similar to that activated by sodium taurocholate and to that specifically precipitated by antibodies specific for BSDL. Consequently, this esterolytic activity was representative of the activity of BSDL present in serum. Examination of the concentration of BSDL, as determined by an enzyme-linked immunosorbent assay technique (5), present in normolipidemic patients demonstrates that it did not differ with sex (males: 4.1 ± 2.8 ng/mL; females: 3.3 ± 2.0 ng/mL). As a consequence, the BSDL activity present in serum did not vary with sex (males: 0.11 ± 0.04 U/mL, females: 0.12 ± 0.03 U/mL). In addition, the relationship between BSDL activity and age was not detectable in the male or female population.

Relationship between BSDL activity and lipids in serum. The initial experiments above demonstrated that BSDL activity can be detected in healthy normolipidemic volunteers. Since this enzyme is potentially important for lipid metabolism (9), we investigated the possible relationship between the activity of BSDL and lipids present in serum sample. For this purpose, the activity of BSDL was tentatively correlated to triglyceride, phospholipid, and total cholesterol concentrations in serum. As shown in Figure 1, no correlation was found between BSDL activity and triglyceride ($n = 28$, $P > 0.05$, $y = 1.05 - 1.37x$, $r = 0.17$, see Fig. 1A). Although a highly significant relationship ($n = 39$, $P < 0.001$, $y = 1.04 + 6.09x$, $r = 0.54$) with a positive slope was detected with total cholesterol present in serum samples (Fig. 1A), no significant relationship was found with phospholipids ($n = 28$, $P \approx 0.05$, $y = 6.76\ 10^{-2} + 0.13x$, $r = 0.36$, Fig. 1B). The relationship between BSDL activity and total cholesterol leads us to resolve

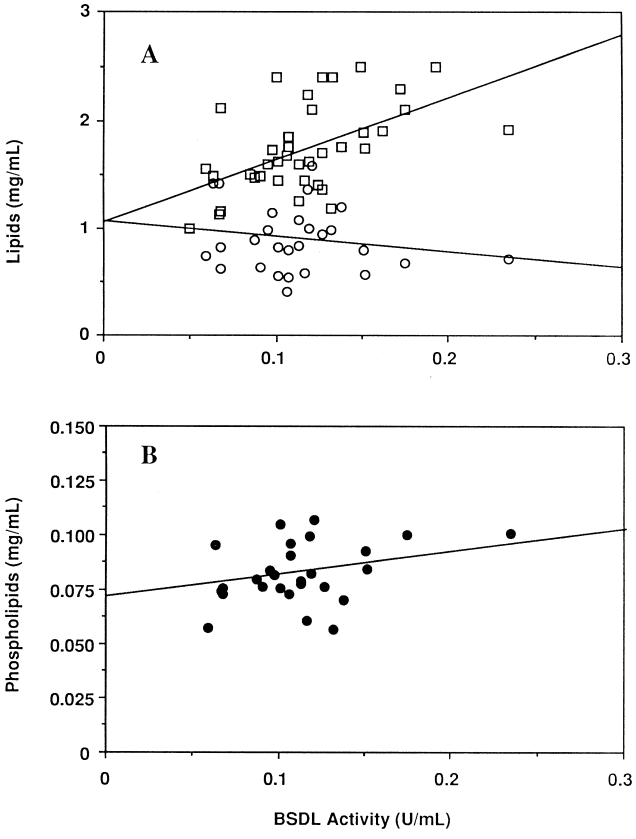

FIG. 1. Relationship between bile salt-dependent lipase (BSDL) activity and lipid concentrations in serum. (A) Total cholesterol (□), triglycerides (○) and (B) phospholipids (●) were determined by adequate methods. The BSDL activity was expressed as the diisopropyl fluorophosphate-inhibited esterolytic activity using 4-nitrophenyl hexanoate as substrate.

whether BSDL activity was linked to either LDL- or HDL-cholesterol. A significant relationship ($n = 35$, $P < 0.01$, $y = 0.51 + 5.50x$, $r = 0.51$) with a positive slope was found between BSDL activity and LDL-cholesterol while no relationship was detected between BSDL activity and HDL-cholesterol ($n = 35$, $P > 0.05$, $y = 0.44 + 0.26x$, $r = 0.09$, Fig. 2). It is worthwhile noting that neither triglycerides, phospholipids, nor total cholesterol present in sera increased significantly with patient age; nevertheless, a poorly significant increase was observed with LDL-cholesterol ($n = 28$, $P < 0.05$, $y = 0.67 + 5.70\ 10^{-3}x$, $r = 0.42$). None of these lipid concentrations was linked to the sex of our donor population.

Relationship between BSDL activity and Apo in serum. We went further in this study in examining possible relationships between BSDL activity and Apo present in serum as suggested by the relationship between BSDL activity and LDL-cholesterol. Eventual linkages between Apo A-I and Apo B100 with sex and age were investigated. Concerning sex, no significant difference was found with Apo A-I concentration (males: 1.32 ± 0.52 mg/mL; females: 1.40 ± 0.32 mg/mL) or Apo B100 concentration (males: 0.98 ± 0.29 mg/mL; females: 0.94 ± 0.34 mg/mL). Also, no relationship was detected between age and Apo A-I concentration. Nevertheless, Apo B100 increased a little with age ($n = 28$, $P < 0.05$, $y = 0.71 + 3.55\ 10^{-3}x$, $r = 0.38$). We next attempted to relate BSDL activity to Apo A-I and Apo B100 concentrations. A significant relationship associated with a positive slope was found between BSDL activity and Apo B100 ($n = 40$, $P < 0.01$, $y = 0.39 + 4.71x$, $r = 0.44$, Fig. 3) or Apo A-I ($n = 40$, $P < 0.01$, $y = 0.82 + 4.89x$, $r = 0.44$).

Binding of BSDL to LDL. In previous publications, we showed that BSDL activity on cholesteryl esters and triglycerides was inhibited by LDL but not by lipoprotein-depleted plasma (6). We also showed that BSDL was able to degrade

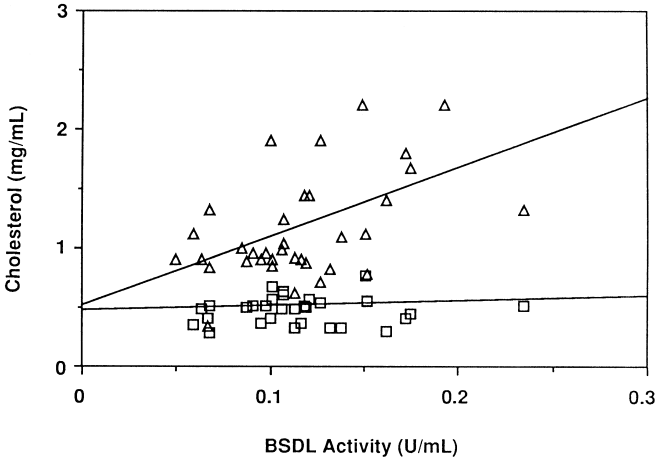

FIG. 2. Relationship between BSDL activity and lipoprotein cholesterol. High density lipoprotein (□) and low density lipoprotein (△) were isolated by specific precipitation and their total cholesterol content determined by enzymatic methods. The BSDL activity was evaluated as described in the legend of Figure 1. See Figure 1 for other abbreviation.

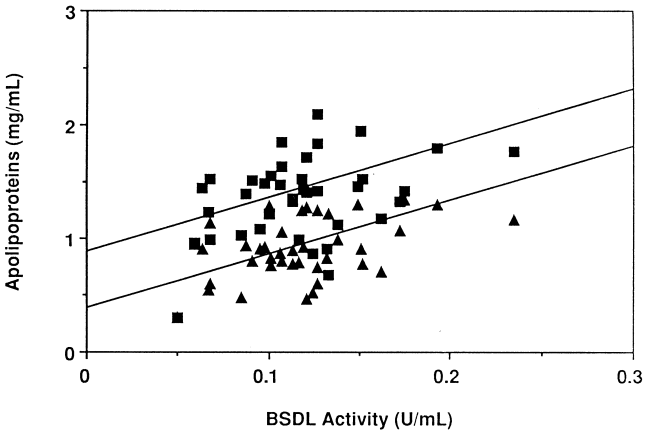

FIG. 3. Relationship between BSDL activity and apolipoprotein (Apo) level. Apolipoprotein levels were evaluated by the turbidity method using specific antibodies to Apo A-I (■) or Apo B100 (▲). BSDL activity was measured as described above in the legend of Figure 1. See Figure 1 for other abbreviation.

oxidized LDL (9). In light of the results presented above, these data suggested that BSDL may have a specific interaction with LDL. To detect such an interaction, ^{125}I-BSDL (4 pmoles) was incubated with a preparation of lipoproteins, including VLDL, LDL and HDL, and chromatographed by FPLC on a Superose 6 column. Under the conditions used, approximately 70% of the ^{125}I-BSDL was coeluted with LDL while 20% was associated with VLDL. Only a minute amount of ^{125}I-BSDL was detected in HDL-containing fractions (not shown). These data indicated that BSDL may be specifically associated with LDL. Nevertheless, this may indicate that BSDL recognizes LDL as a potential substrate.

Interaction between BSDL and Apo B100. In another series of independent experiments, Apo B100 and Apo A-I, isolated from human LDL and HDL, were analyzed on SDS-PAGE and Western-blotting using *pAb L64* antibodies specific for human pancreatic BSDL as primary antibodies. As shown in Figure 4A, BSDL could be detected in Apo B100, but not in Apo A-I preparations. This result, which was reproduced with preparations of Apo originating from many different normolipidemic sera, suggests that BSDL in serum is associated with Apo B100 but not Apo A-I. In addition, we determined whether BSDL was capable of a direct interaction with Apo. For this purpose, ^{125}I-labeled BSDL (≈ 50,000 cpm, approximately 1 pmole) was incubated with 1 nmole of Sepharose-immobilized Apo B100 or 5 nmoles of Sepharose-immobilized Apo A-I. After an overnight incubation and an exhaustive washing of beads and correction for nonspecific binding to control agarose beads, more than 82% of the loaded radioactivity remained associated with Sepharose-immobilized Apo B100 while no radioactivity was bound to Sepharose-immobilized Apo A-I beads. The ^{125}I-BSDL bound to Sepharose-immobilized Apo B100 could have been displaced by increasing amounts of unlabeled BSDL (Fig. 4B) which supports the specificity of the interaction. The

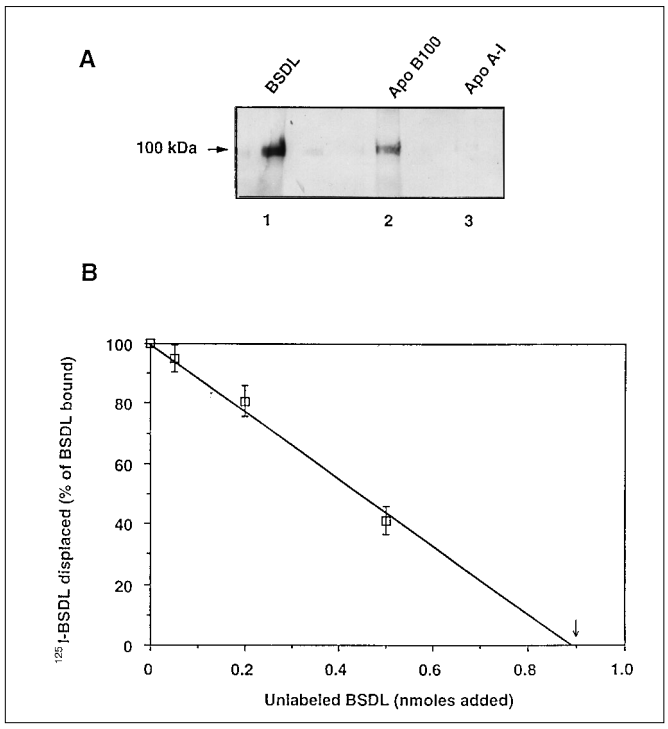

FIG. 4. Association of BSDL with Apo B100. (A) Apo B100 and Apo A-I were isolated by centrifugation over a KBr density gradient and analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were then electrotransferred onto a nitrocellulose membrane and probed with antibodies specific for human pancreatic BSDL (*pAb L64*). Lane 1, pure BSDL (0.7 ng loaded); lane 2, Apo B100 (15 µg); lane 3, Apo A-I (15 µg). (B) Displacement of ^{125}I-BSDL bound to Sepharose-immobilized Apo B100. ^{125}I-BSDL was firstly incubated with Sepharose-immobilized Apo B100, the bound radiolabeled protein (which represents more than 80% of loaded material) and then was displaced with increasing amounts of unlabeled BSDL as indicated. Extrapolation of the regression line ($y = 101.5 - 118.3x$, $r = 0.997$) to the origin gives the amount of unlabeled BSDL (0.87 nmole, arrow) required for the complete displacement of radioactive BSDL. See Figures 1 and 3 for abbreviations.

complete displacement of ^{125}I-labeled BSDL could be achieved with 0.87 ± 0.07 nmoles of unlabeled BSDL. Because 1 nmole of Sepharose-immobilized Apo B100 was used in these experiments, one may suggest that the stoichiometry of the binding of BSDL to Apo B100 was 1:1. These data suggest that in LDL one molecule of Apo B100 may be able to bind one molecule of circulating BSDL.

DISCUSSION

Besides its role in lipid digestion, BSDL which transits through the enterocyte, was demonstrated to exert a systemic action in serum on oxidized LDL (9). Oxidized LDL is enriched in lysophosphatidylcholine which is thought to mediate deleterious effects on vascular endothelium (12). Because oxidation of LDL occurs after LDL enters the subintimal space, to hydrolyze lysophosphatidylcholine, the best location for BSDL to exert its function would be arterial tissue. In human aorta, BSDL immunoprecipitated by specific antibodies to pancreatic BSDL was characterized (9). The source of arterial pancreatic-like BSDL could be multiple. It could originate from the circulating pool derived from the pancreas (4, 5), or from circulating cells such as eosinophils (21). Lastly, aortic BSDL may be synthesized by the vascular endothelial cells.

It is clear that given its ability to hydrolyze lysophospholipids (1), pancreatic-like BSDL, independent of its origin, could be a protective factor against atherogenesis (9). Therefore, it appeared natural to examine whether a linkage between circulating BSDL and serum lipids exists. Recent studies suggested that at least two isoforms of BSDL were secreted by the pancreas (22), one of them being 10 times more active on cholesteryl esters and 4-nitrophenyl hexanoate (23). Because *pAb L64* does not discriminate between these two isoforms of BSDL, the serum level of BSDL determined by enzyme-linked immunosorbent assay cannot be directly correlated with enzyme activity. To overcome this fact, we attempted to find a rapid and convenient way to detect BSDL activity in serum. For this purpose the esterolytic activity of serum was investigated in the absence of a BSDL activator (i.e., sodium taurocholate) or after treatment of serum with a BSDL inhibitor (i.e., DFP) and subtracted from values obtained in the presence of the BSDL activator or before treatment with the inhibitor. The difference represents the serum esterolytic activity which is bile salts-activated and DFP-inhibited, respectively. In both cases, this esterolytic activity was similar. Two other lipases, the lipoprotein-lipase (LPL) and hepatic lipase released into plasma on intravenous injection of heparin, may present esterolytic activity (24,25). LPL and hepatic lipase are distinct enzymes but have very similar hydrolytic functions. They both hydrolyze lipoprotein triglycerides and are potentially active on water-soluble substrates such as 4-nitrophenyl butyrate (26). Although both LPL and hepatic lipase are inhibited by serine protease inhibitors (27,28), the contribution of these two enzymes to the esterolytic activity in serum appeared very low in the absence of intravenous heparin administration to patients (29,30). The fact that the fraction of esterolytic activity, which is inhibited by DFP, was within experimental errors similar to that activated by bile salts and to that immunoprecipitated by polyclonal antibodies specific for BSDL, suggested that the fraction of DFP-inhibited esterolytic activity determined on 4-nitrophenyl hexanoate was representative of the BSDL activity in serum. Under these conditions, BSDL activity was related to cholesterol and particularly to LDL-cholesterol as BSDL activity increased with increasing amounts of LDL-cholesterol. A significant relationship was also detected between BSDL activity and Apo B100 concentration in serum. Indeed, in light of the increasing activity of BSDL with LDL-cholesterol, the relationship with Apo B100 was expected. The question then was to determine whether BSDL was able to interact with LDL and in part with Apo B100. Indeed, data presented in the study indicated that, first ^{125}I-BSDL coeluted in gel filtration with LDL, second BSDL can be detected in Apo B100 preparations, and third BSDL can bind to Apo B100 in

a 1:1 stoichiometry. Consequently, it is suggested that BSDL is able to bind to circulating LDL by a specific interaction with Apo B100. This agreed with previous data indicating that BSDL activity on cholesteryl esters can be inhibited by LDL (6) and that BSDL was able to degrade oxidized LDL (9). Because BSDL transcripts were not detected in normal human adult tissues, other than pancreatic and mammary glands (31), these results suggested that BSDL found in endothelial vascular tissue (32) may originate from the LDL-associated BSDL. Since oxidation of LDL is thought to occur within the endothelium of the arterial wall (33), the LDL-associated BSDL may have the correct intracellular location to degrade oxidized LDL, once captured by aortic endothelial cells.

In conclusion, it is shown for the first time that BSDL activity may be detected in serum and that this activity increases with LDL-cholesterol. It is also shown, for the first time, that circulating BSDL may be partly associated with LDL and may have a specific interaction with Apo B100. Taken as a whole with previously published data (9), it is strongly suggested that pancreatic-like serum BSDL may have positive effects against the atherogene processes.

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Epicoprostanol Found in Adipocere from Five Human Autopsies

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ABSTRACT: Adipocere formation is well known as a later postmortem change. We collected adipocere from five male victims which had been submerged under the sea or fresh water for 1 mon to 4 yr. Fresh subcutaneous fat of a male victim who died from a cerebral contusion was used as the control. The samples were homogenized, and the lipids were extracted with chloroform and methanol followed by injection into a gas chromatograph and a gas chromatograph–mass spectrometer. We detected hydroxy fatty acids (10-hydroxyoctadecanoic acid and 10-hydroxyhexadecanoic acid) as well as 10-ketooctadecanoic acid in adipocere, but not in the control. In addition, we found for the first time a cholesterol-related peak with a molecular ion of 388 in adipocere and identified it as epicoprostanol, suggesting not only oxidation but also reduction had occurred during the formation of adipocere. In addition, we showed the time-course of epicoprostanol accumulation. The relationship between the time of adipocere formation and the characteristic lipid composition is discussed.
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Adipocere is one of the later postmortem changes. It is seen in bodies immersed in water or in damp and warm environments. It is caused by hydrolysis and hydration of adipose tissue, leading to the formation of a greasy or waxy substance. Adipocere is said to take several months to develop, although development can be as short as several weeks. Adipocere contains palmitic, oleic, and stearic acids (1,2). Investigations have shown that adipocere contained 10-hydroxyoctadecanoic and 10-hydroxyhexadecanioc acids (3–5) as well as 10-ketooctadecanoic and 10-ketohexadecanoic acids (5,6).

In the present study, we collected adipocere samples from five victims who had been submerged in water from 1 mon to 4 yr. The lipids obtained for the adipocere samples were analyzed by gas chromatography (GC) and GC–mass spectrometry (MS) to determine if the composition of the various lipids in the adipocere is dependent upon the length of the period of immersion of the victim after death.

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Abbreviations: GC, gas chromatography; GC–MS, GC-mass spectrometry; IS, internal standard; Rt, retention time; TIC, total ion chromatogram.

MATERIALS AND METHODS

Subjects. Selected case reports are presented below.

Case 1: This 44-year-old man was found dead in a fisherman's net in the sea 1 mon after he was reported missing. The cause of death was drowning. Adipocere was partially formed.

Case 2: This 61-year-old man was found dead by a fisherman in an irrigation channel 2 mon after he was reported missing. The cause of death was unknown. Adipocere was partially formed.

Case 3: This 49-year-old man was found dead in his car which was found sunk in a pond 4 mon after he was reported missing. The cause of death was estimated drowning. Adipocere was formed on almost the entire body.

Case 4: This man in his forties was found dead in a fisherman's net in the sea 4 to 6 mon after he was reported missing. The cause of death was estimated drowning. Adipocere was formed on almost the entire body.

Case 5: This 23-year-old man was found dead in his car which was found sunken in the sea 4 yr after he was reported missing. The cause of death was unknown. Skeletonization of almost the entire body was seen, whereas the remains formed adipocere.

Control: This 36-year-old man fell in front of his house and died from a cerebral contusion. The abdominal subcutaneous fat tissue was collected during the autopsy 15 h after his death.

Chemicals. Methyl esters of *cis*-11-eicosenoic (20:1) acid and hentriacontanoic (31:0) acid were purchased from Sigma (St. Louis, MO). Cholesterol, cholestanol, epicholestanol, coprostanol, and epicoprostanol were purchased from Steraloids, Inc. (Wilton, NH). Hydrogen chloride (5%, wt/vol) in methanol was obtained from Nachalai Tesque, Inc. (Kyoto, Japan). 10-Hydroxyoctadecanoic acid was provided by Prof. Takatori of Tokyo University. The methyl ester was obtained by transmethyl-esterification with diazomethane. All other chemicals were of analytical grade.

Extraction. Adipocere (0.2 to 0.5 g) and the subcutaneous fat from the control were weighed, minced with scissors, homogenized, and extracted at room temperature essentially

by the method of Folch *et al.* (7). The chloroform was evaporated under a nitrogen stream. The crude lipids were placed in a screwcap-sealed reactivial with hydrogen chloride in methanol and heated at 110°C for 1 h to convert the lipids into their methyl esters. Methanol was evaporated under a nitrogen stream, and the lipid residue was redissolved in chloroform.

GC–MS and GC. A 1 μL aliquot of the sample was injected into a DB-1 fused-silica megabore column (30 m × 0.53 mm i.d., film thickness 1.5 μm) (J&W Scientific, Folsom, CA) which was mounted in a Model QP5000 Shimadzu GC–MS (Kyoto, Japan). The following chromatographic conditions were used. Helium was used as the carrier gas at a flow rate of 20 mL/min. The column temperature was 140°C for 2 min, then programmed to 310°C at 4°C/min, and finally maintained at 310°C for 10 min. The injection port temperature was 240°C and the separator temperature 250°C. The ionization voltage was 70 eV, ionization current 60 μA, and scan interval 0.5 s. All peaks corresponding to the methyl esters of the fatty acids were identified by their retention times (Rt) and mass spectra. Quantitative determinations by selected ion monitoring were carried out. The following ions were focused on: *m/z* 215, fragment ion of epicoprostanol; *m/z* 386, M^+ ion of cholesterol; and *m/z* 143, fragment ion of an internal standard (IS, 31:0). Standard curves were prepared by selected ion monitoring analyses of 10, 20, and 40 μg/mL of epicoprostanol and cholesterol with 50 μg/mL of IS. The peak areas of the ions monitored were measured, and the ratios of the fatty acids to the IS were computed for the standard compounds and for the lipid extracts of the specimens.

The GC was a Shimadzu GC-7A equipped with a flame-ionization detector. The esters were separated on a Omegawax TM 530 fused-silica megabore column (30 m × 0.53 mm i.d., film thickness 0.5 μm) (Supelco, Inc., Bellefonte, PA). The following chromatographic conditions were used. Helium was used as the carrier gas at a flow rate of 20 mL/min. The column temperature was 150°C for 1 min, then programmed to 270°C at 8°C/min, and finally maintained at 270°C for 2 min. The injection and detector temperature were 260°C. The individual peak areas of the fatty acids were calculated by a Model C-R6A chromatopac (Shimadzu).

RESULTS

Figure 1 shows the total ion chromatograms (TIC) for adipocere and normal adipose tissue by GC–MS. The lipids in both the control and the adipocere contained fatty acids [main components: myristic (14:0), palmitoleic (16:1), palmitic (16:0), linoleic (18:2), oleic (18:1), and stearic (18:0)]. In the adipocere peaks 1, 2, and 4 appeared near peak 18:0, and peak A appeared before cholesterol.

Peak A in Figure 1 was subjected to electron-impact MS. Mass spectra of peak A, standard epicoprostanol, coprostanol, cholestanol, and epicholestanol are shown Figure 2. Peak A has a molecular ion (M^+) at *m/z* 388 which is consistent with

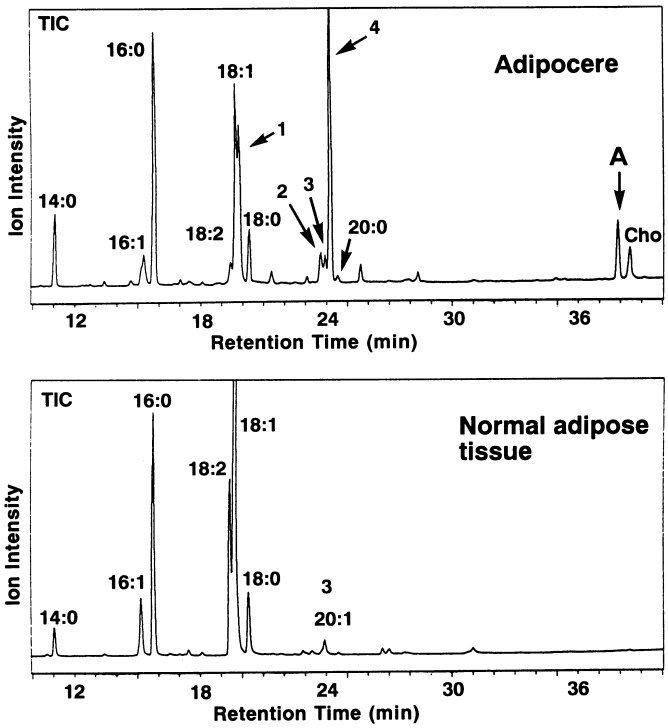

FIG. 1. Total ion chromatograms (TIC) for adipocere and normal adipose tissue (control). 1, 10-Hydroxyhexadecanoic acid (10-OH 16:0); 2, 10-ketooctadecanoic acid (10-keto 18:0); 3, eicosenoic acid (20:1); 4, 10-hydroxyoctadecanoic acid (10-OH 18:0); A, epicoprostanol; Cho, cholesterol.

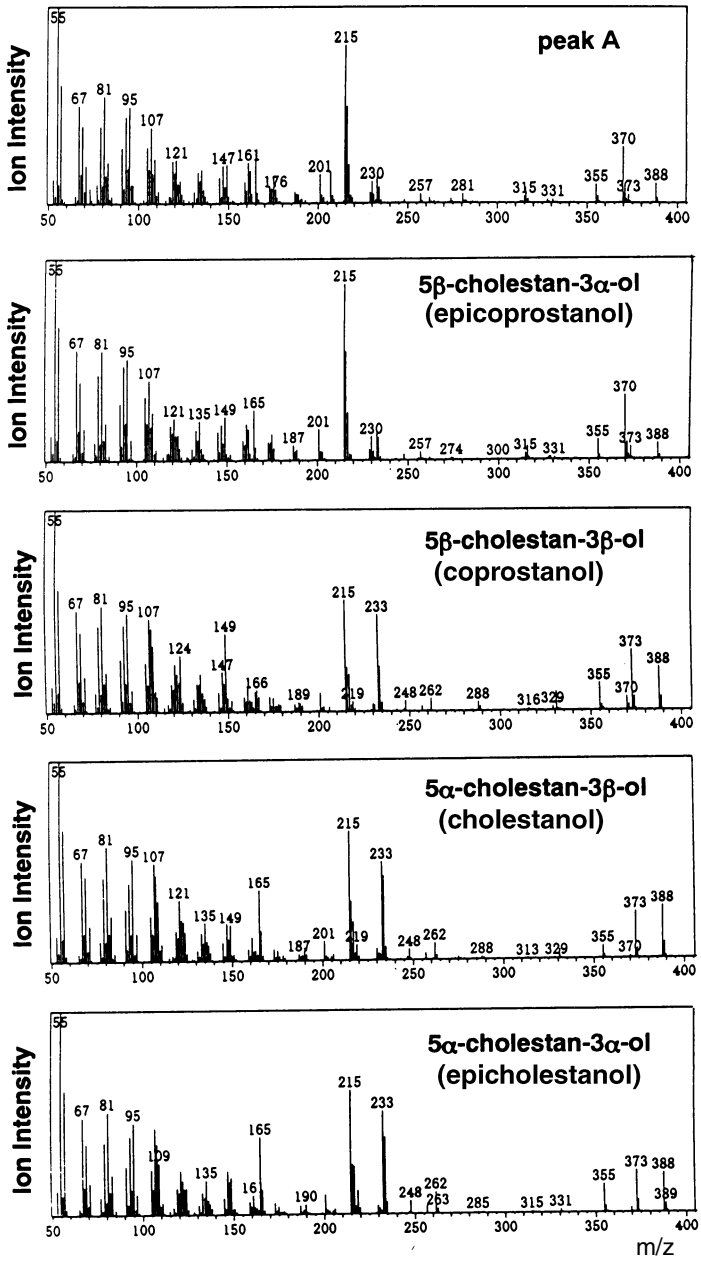

FIG. 2. Electron-impact mass spectra of peak A from adipocere and standard epicoprostanol, coprostanol, cholestanol, and epicholestanol.

epicoprostanol, the reduced compound of cholesterol. Figure 3 shows TIC for standard epicoprostanol with IS (31:0) and adipocere with IS. The Rt of peak A was consistent with that of epicoprostanol. Mass chromatograms at *m/z* 215, 370, 230, and 388 for the adipocere and the control are also shown in Figure 3. No peaks appeared in monitoring at *m/z* 215, 370, and 388 in the control sample. The Rt and the ratio of the ion intensity at *m/z* 215 to 233 of four stereoisomers of 5-cholestan-3-ol are shown in Table 1. Thus, peak A was identified as epicoprostanol.

The levels of epicoprostanol and cholesterol were determined by selected ion monitoring, and the ratio of epicoprostanol to cholesterol in the adipocere samples is shown in Table 2. The ratio increased as the period after death increased.

Next, peaks 1, 2, 3, and 4 were subjected to electron-impact MS. As shown in Figure 4, peak 2 with fragment ions at *m/z* 214, 199, 157, and 156 was assumed to be 10-ketooctadecanoic acid because these were the characteristic ions reported by Takatori and Yamaoka (3). Peak 3, appearing at Rt of 23.9 min with fragment ions at *m/z* 292 and 250 and M^+ ion at *m/z* 324, was consistent with standard 20:1. Peak 4, appearing at Rt of 24.3 min with fragment ions at *m/z* 169, 172, and 201, was consistent with standard 10-hydroxyoctade-

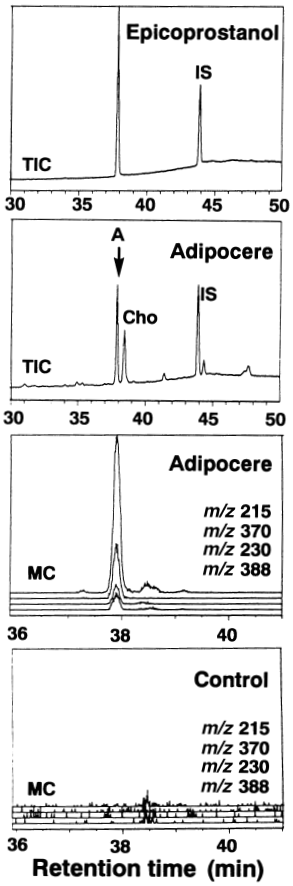

FIG. 3. TIC between 30 and 50 min for standard epicoprostanol with internal standard (IS, 31:0) and adipocere with IS. Mass chromatograms (MC) with monitoring at *m/z* 215, 370, 230, and 388 from adipocere and control. See Figure 1 for abbreviations.

canoic acid (*m/z* 169, 172, and 201). Thus, peak 4 was identified as 10-hydroxyoctadecanoic acid.

TIC from 19 to 21.5 min in control and adipocere are shown in Figure 5. Mass chromatogram 1 in adipocere at *m/z* 201, 172, 169, and 143, being the characteristic ions to 10-hydroxyhexadecanoic acid, is also shown in Figure 5. Thus, peak 1 was assumed to be 10-hydroxyhexadecanoic acid. Mass chromatogram 2 at *m/z* 128, 157, and 214, being the characteristic ions of 10-ketohexadecanoic acid reported by Takatori and Yamkaoka (6), is shown in Figure 5. It was possible that the peaks indicated by C were due to 10-ketohexadecanoic acid.

TABLE 1
Four Stereoisomers of 5-Cholestan-3-ol

	5	3	Rt (min)	215/233[a]
Stereoisomers				
Cholestanol	α	β	38.57	1.36
Epicholestanol	α	α	38.51	1.20
Epicoprostanol	β	α	37.86	5.38
Coprostanol	β	β	37.85	1.18
Peak A			37.86	5.33

[a]Ratio of ion intensity at *m/z* 215 to *m/z* 233. Rt, retention time. Column of gas chromatography–mass spectrometry: DB-1 (J&W Scientific, Folsom, CA).

TABLE 2
Levels of Epicoprostanol (A) and Cholesterol (B) in Five Adipoceres

Case	Period (mon)	Epicoprostanol (μg/g[a])	Cholesterol (μg/g[a])	Ratio (A/B)
1	1	18	185	0.10
2	2	71	597	0.12
3	4	326	286	1.14
4	4–6	173	88	1.97
5	48	154	74	2.08

[a]Values represent μg per gram of wet tissue.

Figure 6 shows the gas chromatogram of methyl esters of fatty acids in adipocere. To separate peaks of 20:1, 10-ketooctadecanoic acid and 10-hydroxyoctadecanoic acid, we used different column in GC–MS. The peak for 10-ketooctadecanoic acid was assumed by comparison with TIC of GC–MS. The fifth case (the 23-year-old man in the sea 4 yr) was used for Figures 1–6.

The fatty acid composition of the five adipocere samples and the control is shown in Table 3. The control, Case 1, and Case 2 which do not contain 10-ketooctadecanoic acid have 18:2 present greater than 10%. The amount of 18:2 was much

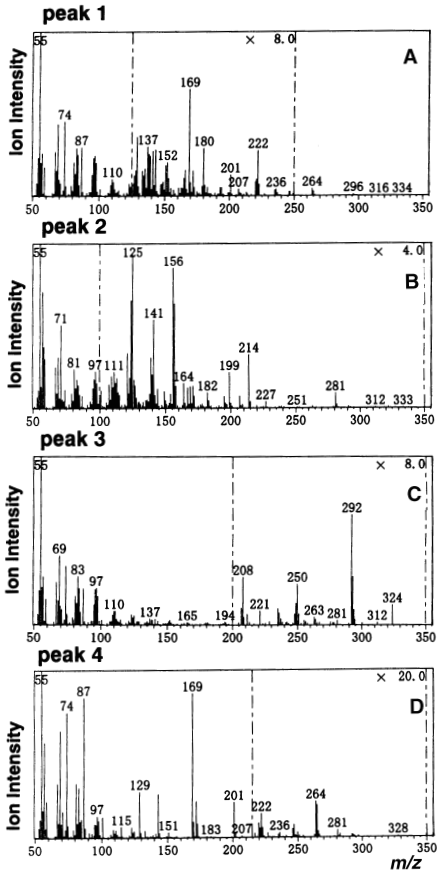

FIG. 4. Electron-impact mass spectra of peaks 1, 2, 3, and 4 from adipocere. (A) 10-OH 16:0, 10-Hydroxyhexadecanoic acid; (B) 10-keto 18:0, 10-ketooctadecanoic acid; (C) 20:1, *cis*-11-eicosenoic acid; (D) 10-OH 18:0, 10-hydroxyoctadecanoic acid.

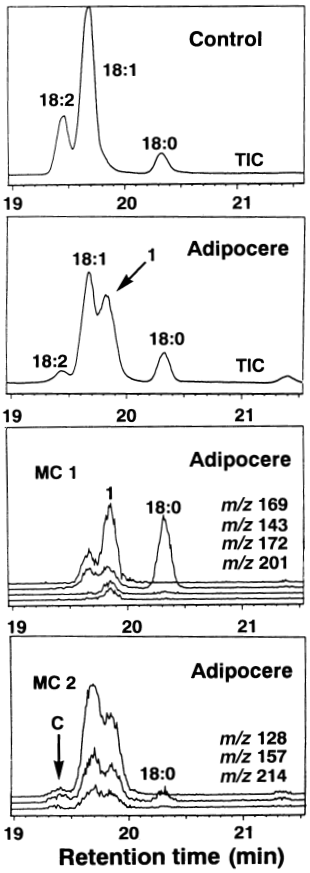

FIG. 5. TIC between 19 and 21.5 min for control and adipocere. MC for adipocere with monitoring at *m/z* 169, 143, 172, 201, and at *m/z* 128, 157, and 214. 1, 10-Hydroxyhexadecanoic acid; C, 10-ketohexadecanoic acid. See Figures 1 and 3 for other abbreviations.

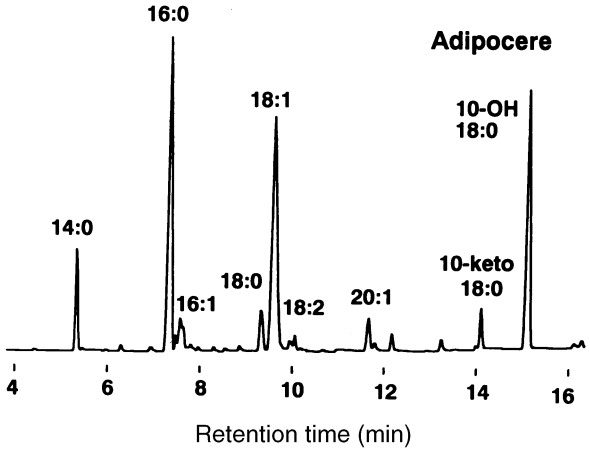

FIG. 6. Gas chromatographic profiles of methyl esters of fatty acids in adipocere. Column of gas chromatography: Omegawax TM530 (Supelco, Inc., Bellefonte, PA).

less in Cases 3, 4, and 5 which contained 10-ketooctadecanoic acid. The amount of 10-hydroxyoctadecanoic acid increased as the length of time the victim was submerged in water increased.

DISCUSSION

The results of the present study show for the first time that the adipocere of the victims contained significant amounts of epicoprostanol, whereas this compound was essentially absent from normal adipose tissue. The structure was confirmed by the mass spectrum on GC–MS.

Coprostanol is the major metabolite of cholesterol in the intestine by the bacterial microflora (8). The relative amounts of cholesterol and its metabolite found in human feces are as follows: cholesterol makes up about 20% of the neutral sterol concentration; coprostanol, 65%; coprostanone, 10%; and cholestanol, cholestanone, and epicoprostanol, collectively about 5% of the neutral sterols in feces (9). Only a trace amount of epicoprostanol has been found in human feces. Korpela (10) analyzed fecal samples from eight healthy young men who were on a normal Finnish diet and found epicoprostanol in only one sample. Accordingly, little is known about the occurrence of epicoprostanol in humans. An elevated cholestanol level was found in serum of patients with cerebrotendinous xanthomatosis, a hereditary disorder (11).

Epicoprostanol formation is considered to occur as follows. 4-Cholesten-3-one is formed by the oxidation of the 3β-hydroxy group of cholesterol to a ketone and isomerization of the 5-6 double bond to the 4-5 position. Coprostanone is formed by the reduction of the 4-5 double bond. Subsequently, coprostanol and epicoprostanol are formed by the reduction of the 3-keto group to the 3β-hydroxy and 3α-hydroxy groups, respectively. As we did not find cholestanol, epicholestanol, and coprostanol, we propose that in adipocere

TABLE 3
Fatty Acid Compositions in Adipoceres and a Control[a]

Subject	Period	14:0	16:0	16:1	18:0	18:1	18:2	20:1	10-Keto -18:0	10-OH -18:0
Control	15 h	1.8	20.0	4.9	3.6	42.8	11.7	1.3	—	—
Case 1	1 m	1.3	14.2	6.2	1.6	36.4	15.1	2.0	—	0.6
2	2 m	2.1	11.4	8.2	3.5	42.3	13.3	1.3	—	1.2
3	4 m	5.4	47.6	4.8	4.2	9.8	0.8	1.5	0.7	14.7
4	4–6 m	2.3	7.0	4.9	2.2	52.1	6.2	5.3	2.5	11.6
5	4 yr	5.5	25.1	4.6	3.4	27.7	2.1	2.8	3.2	22.2

[a]Values are expressed as percentage by weight of total fatty acids. Period: postmortem time of samples.

epicoprostanol is more easily produced than the other metabolites of cholesterol.

Next, some authors have postulated the sequence leading to adipocere formation (4,6,12–14).

Tomita (4) showed that hydroxy and keto fatty acids in adipocere of mice rose to 60% (maximum) in only 2 mon and fell to 10% in 6 mon as adipocere formation reached completion. In contrast, Takatori and Yamaoka (6) reported that the relative amount of hydroxy fatty acids was 3% in adipocere of a victim immersed for 3 mon, while in two adipocere samples immersed for 6 mon, the amount of fatty acids including keto fatty acids reached about 20%. In the present study of adipocere of two victims immersed for 1 mon and 2 mon, the amount of hydroxy fatty acid was only 1%, but the amounts of 18:2 were 13 and 15%. In the adipocere of three victims immersed from 4 mon to 4 yr, the amounts of hydroxy fatty acids rose from 14 to 22%, but those of 18:2 fell to only several percentage. Consequently, our results regarding hydroxy fatty acid composition were not consistent with the results of Tomita (4), but were similar to those of Takatori and Yamaoka (6). Both of them failed to show hydroxy and keto fatty acids as well as ordinary fatty acids in the same chromatogram, whereas we successfully demonstrated them by GC. Furthermore, we showed that in the adipocere of the victims immersed for less than 3 mon, keto fatty acids did not develop, and that the ratio of epicoprostanol to cholesterol was 0.1, whereas the ratio rose from 1.1 to 2.1 in the adipocere of three victims immersed over 4 mon when adipocere formation seemed to be complete. Thus, the analysis of epicoprostanol might be of value to determine the time of death.

Finally, we clarified the relationship of the characteristic lipids in adipocere to the total time of immersion by comparing the composition of adipocere from the five victims.

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Prevention of Ischemia-Induced Cardiac Sudden Death by n-3 Polyunsaturated Fatty Acids in Dogs

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ABSTRACT: The objective of this study was to obtain functional information associated with the prevention by n-3 polyunsaturated fatty acids (PUFA) of ischemia-induced fatal cardiac ventricular arrhythmias in the intact, conscious, exercising dog. Thirteen dogs susceptible to ischemia-induced ventricular fibrillation were prepared surgically by ligation of their anterior descending left coronary artery and placement of an inflatable cuff around their left circumflex artery. After 4 wk of recovery, exercise-plus-ischemia tests were performed without and then with an intravenous infusion of an emulsion of free n-3 PUFA just prior to occluding the left circumflex artery while the animals were running on a treadmill. One week later the exercise-plus-ischemia test was repeated but with a control infusion replacing the emulsion of n-3 PUFA. The infusion of the free n-3 PUFA in quantities of 1.0 to 10 g prevented ventricular fibrillation in 10 of the 13 dogs tested ($P < 0.005$), apparently without esterification of the PUFA into membrane phospholipids. The antiarrhythmic effect of the n-3 PUFA was associated with slowing of the heart rate, shortening of the QT-interval (electrical action potential duration), reduction of left ventricular systolic pressure, and prolongation of the electrocardiographic atrial-ventricular conduction time (P-R interval). These effects are comparable with those we have reported in studies with cultured neonatal rat cardiac myocytes. *Lipids 32*, 1161–1168 (1997).

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Abbreviations: DHA, *cis*-5,8,11,14,17,20-docosahexaenoic acid (22:6n-3); ECG, electrocardiogram; EPA, *cis*-5,8,11,14,17-eicosapentaenoic acid (20:5n-3); NEFA, nonesterified fatty acids [fraction of plasma lipids which includes the free fatty acids of which over 99.9% are bound to albumin and other plasma proteins plus a very small fraction of monomeric free or aggregated clusters of fatty acids (lamellar or micellar aggregates) in plasma water]; P-R interval, duration of conduction time from the start of the auricular P-wave to start of the ventricular R-wave conventionally measured from the ECG; PUFA, polyunsaturated fatty acids (denotes the n-3 class of essential long-chain PUFA, but more generally applied to both the n-3 and n-6 classes); QT interval, an electrocardiographic interval from the start of the Q-wave to the start of the T-wave of the ECG (equivalent to the duration of the action potential observed in individual cardiac myocytes as an abrupt rise and fall in the transmembrane potential when their resting or diastolic membrane potential is depolarized and measured with an intracellular electrode and an external reference electrode; the electrical signal that initiates contractions of the heart muscle; TLC, thin-layer chromatography; VF, ventricular fibrillation, a fatal cardiac arrhythmia when sustained, characterized by grossly irregular, rapid, and chaotic beating; VT, ventricular flutter, a very rapid but regular beating rate which is also fatal if sustained.

Coronary heart disease is the leading cause of death in all Western industrialized countries. Despite considerable improvement since the mid-1960s, almost half a million individuals die yearly in the United States of America from myocardial infarctions, and 50 to 60% of these die within 1 h, mostly from ventricular arrhythmias (1). Thus, a safe and simple intervention that would prevent ischemia-induced lethal ventricular arrhythmias would have considerable public health benefit.

Based on sporadic earlier suggestions that the n-3 long-chain polyunsaturated fatty acids (PUFA) might have antiarrhythmic effects (2,3), McLennan *et al.* (4,5) have shown that feeding rats a diet in which saturated fat or monounsaturated fat (olive oil) was the major fat source was associated with a high incidence of lethal ventricular arrhythmias upon subsequent coronary artery ligation. In contrast, diets in which a vegetable oil, sunflower seed oil, was the major fat significantly reduced the arrhythmias, while tuna fish oil prevented the ventricular fibrillation (VF). They have supported these findings in nonhuman primates (6).

In 1994 we reported initial confirmatory findings in surgically prepared dogs susceptible to ischemia-induced VF in which seven of the eight animals tested were protected from the expected VF (7). In this group of animals, the fish oil fatty acids were infused as an emulsion intravenously just prior to the ischemic challenge. This route of administration was chosen purposely. If prevention of the arrhythmias occurred, then we considered this would provide convincing evidence that some ingredient(s) of the infused fish oil concentrate was the active agent. In the earlier feeding studies there was, at the time, considerable disagreement as to what dietary change was protective. The present report enlarges the number of animals studied and reports associated physiologic changes, which will be important in the understanding as to how the prevention of ischemia-induced VF is produced by these essential, dietary PUFA.

METHODS

Experiments were performed on 13 conscious, exercising mongrel dogs (14.5–18.3 kg) which had been previously prepared surgically, as described (8–10). Briefly, the animals were anesthetized and instrumented to measure left circumflex coronary artery blood flow and ventricular electrograms.

A hydraulic occluder was also placed around the left circumflex coronary artery. Finally, the left anterior coronary artery was ligated, producing an anterior wall myocardial infarction. After a period of at least 4 wk to allow the dogs to recover from surgery and the acute effects of the myocardial infarction, the animals ran on a treadmill with the workload increased every 3 min for 18 min or until a target heart rate of 210 beats per min (70% of maximum) was attained. During the last minute of exercise, the cuff was inflated, occluding the left circumflex coronary artery. At the end of 1 min, the treadmill was stopped. The occlusion was maintained for an additional minute. When VF or sustained ventricular flutter (VT) occurred, as soon as consciousness was lost the animals were promptly defibrillated. The brief episodes of ischemia, VF, and defibrillation in the exercise-plus-ischemia tests had no residual effects on cardiac function as evidenced by the normal ventricular electrograms exhibited within minutes after the defibrillation, i.e., no ST (segment of electrocardiogram connecting the S and T waves) changes and a normal sinus rhythm were present. Also in the animals in which left ventricular pressures were measured, no elevation in end diastolic pressure or depression of left ventricular dp/dt occurred. Approximately two-thirds of animals tested were susceptible to prompt development of VF. The resistant animals were excluded from the study. Earlier studies (8–10) had demonstrated that the exercise-plus-ischemia test reliably, and for at least 16 wk after infarction, reproduced the VF response in the susceptible animals.

One week after the control study, the exercise-plus-ischemia test was repeated but just after the infusion of an emulsion of 1.0 to 10.0 g of free n-3 PUFA. Preparation K6000; Pronova-Biocare, Lysaker, Norway, containing 70% n-3 PUFA of which free *cis*-5,8, 1 1,14,17-eicosapentaenoic acid (20: 5n-3, EPA) was 33.9% and free *cis*-5,8, 11,14,17,20-docosahexaenoic acid (22:6n-3, DHA) was 25.5%]. This emulsion was injected slowly intravenously over a period of 40 to 60 min. One week later the same animal had another control exercise-plus-ischemia test performed just as with the fish oil emulsion but with either a saline infusion ($n = 8$) or with soybean oil [10% Intralipid® (Baxter, Deerfield, IL); 100 mL] containing only triglycerides ($n = 5$). Intralipid® is used clinically for parenteral nutrition and contains the identical emulsifying vehicles used for the n-3 fatty acids infusion with its fatty acid content all esterified in triglycerides. Thus, each animal tested served as its own control with a control infusion and exercise-ischemia test before and following the test with infusion of the free nonesterified n-3 PUFA.

The principles governing the care and treatment of animals as expressed by the American Physiologic Society were followed at all times during this study. In addition, the procedures used were approved by the Ohio State University Institutional Animal Care and Use Committee.

Analysis of plasma fatty acids. The fatty acid composition of plasma triglycerides and phospholipids was analyzed as described previously (11). Plasma (250 µL) was extracted with chloroform/methanol (2:1, vol/vol). Samples were sealed under nitrogen and stored at −70°C unti1 thin-layer chromatography (TLC) was performed. For TLC, the samples were dried under nitrogen and reconstituted in 80–100 µL chloroform. TLC plates were activated at 90–100°C for 1 h, and chromatography was run using petroleum ether/ethyl ether/acetic acid (80:20:1, by vol) as the solvent. Bands were made visible with 0.01% 8-anilino-1-naphthalenesulfonic acid, and gel scrapings of the triglyceride and phospholipid fractions were collected for methylation. To obtain fatty acid methyl esters, 1.5 mL boron trifluoride in methanol (14%) and 2 mL hexane were added to the gel scrapings and then heated 1 h at 100°C. After this procedure, 1 mL of water was added and mixed vigorously. Tubes were allowed to stand for at least 30 min before removing the hexane phase. Samples were then dried under nitrogen and reconstituted in 100 µL isooctane, of which 2 µL were analyzed by gas–liquid chromatography. An Omegawax™ column (30 m; Supelco, Bellefonte, PA) was used in a Hewlett-Packard 5890A gas chromatograph (Hewlett-Packard, Avondale, PA). Carrier gas was hydrogen (2.39 mL/min), injected with a split ratio of 1:31. The initial temperature was 165°C for 5 min; then temperature was increased to 195°C at 2.5°C/min and from there to 220°C at 5°C/min. Temperature was held for 10.5 min and then decreased to 165°C at 27.5°C/min. Peaks were identified by comparison with fatty acid standards (Nu-Chek-Prep, Elysian. MN), and area percentage for all resolved peaks was analyzed using a Perkin Elmer M1 integrator (Norwalk, CT). By comparison with areas of nearby standard fatty acid peaks, the molar concentrations of the pertinent fatty acids of plasma triglycerides and phospholipids were derived.

Because the emulsifier used for the n-3 PUFA emulsion contained 625 mg of purified egg yolk lecithin (Avanti Polar Lipids, Alabaster, AL) per 50 mL of 10% emulsion, the fatty acid composition of the egg yolk lecithin was analyzed for its fatty acid content. For each of the common PUFA of interest, its content in the plasma phospholipids was subtracted from the total phospholipid fatty acid content of the plasma to obtain the corrected fatty acid content of the plasma free of that contributed by the infused fish oil emulsions. To calculate the concentration of the egg yolk lecithin added to the plasma, it was estimated that the administered 625 mg per 5.0 g of n-3 PUFA would be distributed over the expected 1000-mL plasma volume of a 20-kg dog.

Statistical analyses. Differences of means were analyzed by analysis of variance; other results were evaluated by the χ^2-test or the Fisher Exact Test, as indicated, when any of four values of the 2 by 2 χ^2 table was smaller than 5. Results are expressed as means ± SEM. A significant difference is accepted if $P < 0.05$.

RESULTS

The infusion of the fish oil emulsion prevented lethal ventricular arrhythmias in 10 of the 13 animals tested, whereas these 13 "susceptible" dogs all developed VT or VF with control

infusions both 1 wk before and 1 wk after the n-3 PUFA trial (Fisher Exact Test, $P = 0\ 000536$). By comparison, of the five dogs tested with the fish oil and soybean emulsion, all five receiving the fish oil were protected and none was protected by the Intralipid (five of five protected, Fisher Exact Test, $P = 0.00397$); of the eight dogs tested with fish oil and saline, five of eight were protected (Fisher Exact Test, $P = 0.0120$). Since none of the 26 control studies with both saline or Intralipid was protected, we have combined the data and report an overall protection rate with $P < 0.005$.

Representative recordings obtained from the same animal during the control and 1 wk later following infusion of the

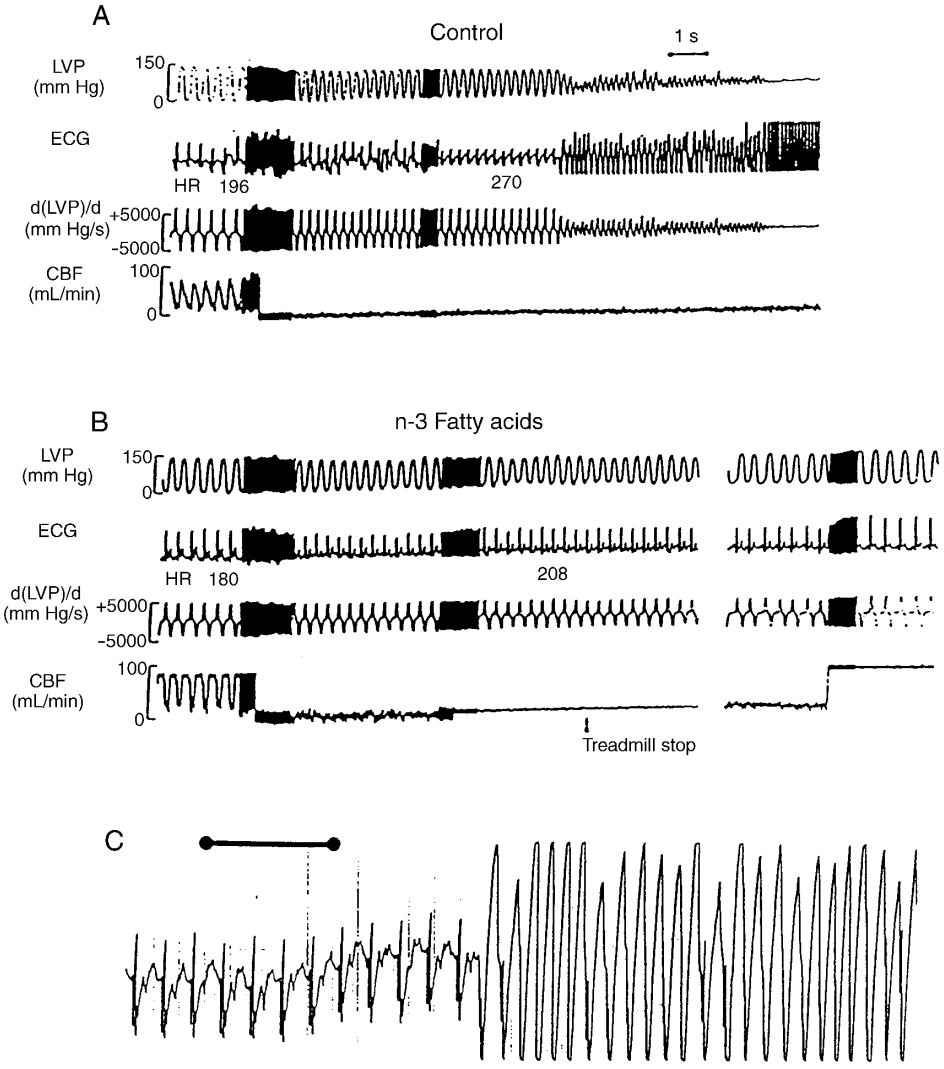

FIG. 1. A representative exercise-plus-ischemia test from the same animal before and after intravenous administration of an emulsion of the n-3 polyunsaturated fatty acid (PUFA). (A) Initial exercise–ischemia control. The dog ran on a treadmill for 18 min with the workload increasing every 3 min. During the last minute of exercise, the cuff was inflated to obstruct blood flow in the left circumflex coronary artery (CBF), as indicated by the abrupt cessation of blood flow in the line marked "CBF." At the end of 1 min of obstruction of the left circumflex artery, the treadmill was stopped and the occlusion was maintained for an additional minute. The chart speed is indicated for 1.0 s (top right), but when the chart speed was slowed twice during the control tracings and three times during the n-3 fatty acid test to condense the tracing, the solid black tracings were recorded. When ventricular arrhythmia occurred, the animal was promptly defibrillated, as soon as consciousness was lost. (B) The repeat exercise–ischemia test 1 wk after the control test following infusion of 5 g of n-3 PUFA intravenously just prior to the exercise–stress test. No arrhythmia occurred, and there were minimal changes in the functional parameters measured despite the obstruction of the CBF. Not shown in this figure was the repeat exercise-plus-ischemia test done 1 wk later in all dogs with either an infusion of 5–7 g of emulsified soybean oil [Intralipid® (Baxter, Deerfield, IL); $n = 5$] or saline ($n = 8$), which in all 13 animals failed to prevent sustained ventricular flutter, or tachycardia (VT) or ventricular fibrillation (VF). LVP = left ventricular pressure; CBF = left circumflex coronary blood flow; d(LVP)/dt was used as an index of the left ventricular contractility. (C) An enlargement of the electrocardiogram (ECG) from Figure 1A showing the arrhythmia that developed with the control exercise–ischemia test in this dog. The arrhythmia is VT, which commenced at a rate of some 270 beat/min and rose to over 400 beats/min by the end of the tracing. At the latter rate it can be seen in A that there was complete circulatory failure as indicated by the cessation of ventricular systolic pulses and of ventricular dP/dt. Such VT occurring in these animals invariably deteriorates into VF, so the dog was promptly defibrillated, as soon as consciousness was lost. The time bar indicates 1.0 s.

fish oil emulsion are displayed in Figure 1. The abrupt drop in the blood flow measured in the left circumflex coronary artery distal to the cuff indicates the time and completeness of occlusion of that artery. The decreases of the left ventricular pressure (LVP) and of the ventricular d (LVP)/dt maximum (an index of ventricular contractility) which occurred with the onset of the ventricular tachycardia seen on the electrocardiogram (ECG) shortly before the treadmill was stopped are evident in the upper control tracings, By contrast, 1 wk later when the identical exercise–ischemia procedure was repeated, but the emulsion of n-3 free fatty acids was infused just prior to the test, the lower tracing shows the minimal changes in these hemodynamic parameters despite the same completeness of the occlusion of the circumflex artery by inflation of the cuff. The second control, 1 wk later, in which the arrhythmic response predictably and reproducibly occurred is not shown since it was the same as this first control. Figure 1C is an enlargement of the tracing of the arrhythmia, VT, experienced by this dog and shows the quite regular beating rate that commenced at a rate of some 270 beats per min and rose to over 400 beats per min by the end of this tracing associated with complete failure of the circulation, as evidenced by the cessation of left ventricular systolic pressure and of left ventricular dP/dt. Our experience is that such ischemia-induced VT deteriorates quickly into VF, so the dog was promptly defibrillated as soon as consciousness was lost. The average time from the occlusion of the left circumflex coronary artery to the onset of VT or VF was 57.4 ± 1.4 s for the first controls and 47.8 ± 8.6 s for the second controls. These data demonstrate that the acute intravenous administration of the free PUFA can prevent the occurrence of malignant ventricular arrhythmias induced by ischemia in this dog model of sudden cardiac death.

The hemodynamic effects of the fish oil emulsion are shown in Table 1 (n = 13 for all comparisons except for the measurements of ventricular systolic pressure and left ventricular dP/dt which were performed on five animals, but with significant changes obtained). The fish oil emulsion significantly attenuated the heart rate in the resting animals (Table 1) and also during exercise (Fig. 2) as well as with the response to the coronary occlusion (control: before, 204.7 ± 9.2, and during occlusion 228 ± 9.2, compared to fish oil: 168 ± 10.6 and 171 ± 15.2 beats/min, respectively). Infusion of the n-3 fatty acids in these dog studies elicited a reduced inotropic effect (decreased left ventricular dP/dt with a reduced ventricular systolic pressure). Table 1 also shows that the PUFA produced a significant shortening of the QT interval (QT is an electrocardiographic interval from the start of the Q-wave to the start of the T-wave of the ECG) corrected for changes in pulse rate (QTc) (12), indicating a shortening of the action potential duration and prolongation of the P-R interval (P-R interval is the duration of conduction time from the start of the auricular P-wave to the start of the R-wave conventionally measured from the ECG). Representative portions of two ECG tracings are shown in Figure 3 to allow visualization of the PR and QT interval changes produced by the PUFA. Both the control strip and the strip following infusion of the n-3 PUFA were taken with the dog in the resting state. The time bars for both strips indicate that 25 mm represents 250 ms. The control heart rate was 107 beats/min and the n-3 PUFA slowed the pulse rate to 40 beats/min, a larger than average slowing effect in this dog. The PR-interval increased from 10 mm or 100 ms to 13 mm or 130 ms, and most of the prolongation occurred during the isoelectric portion of the interval. The QT interval was 150 ms before the infusion and was 170 ms afterward. After correcting for the change in heart rate (12), the QTc-interval values were 200 and 140 ms, respectively. Thus, the n-3 PUFA reduced the QT interval. All

TABLE 1
The Hemodynamic Effects of the Intravenous Infusion of the n-3 PUFA[a]

	Control	Fish oil emulsion	*P*
Resting heart rate (beats/min)	124 ± 4	85 ± 4	<0.001
Ventricular systolic pressure (mm Hg), n = 5	154 ± 6	117 ± 4	<0.001
Left ventricular dP/dt (maximum mm Hg/s), n = 5	5070 ± 353	2960 ± 497	<0.01
QTc interval (ms)	266 ± 7	242 ± 5	<0.02
P-R interval	102 ± 4	122 ± 6	<0.02

[a]The resting heart rate was significantly reduced following infusion of the emulsion containing the n-3 polyunsaturated fatty acids (PUFA). Ventricular systolic pressure and left ventricular dP/dt were determined by a pressure transducer implanted in the left ventricle. Both were significantly reduced by the n-3 PUFA, indicating a reduced inotropic effect. QTc is the OT interval from the electrocardiogram corrected for changes in pulse rate. Note: QT is an electrocardiographic interval from the start of the Q-wave to the start of the T-wave of the electrocardiogram.

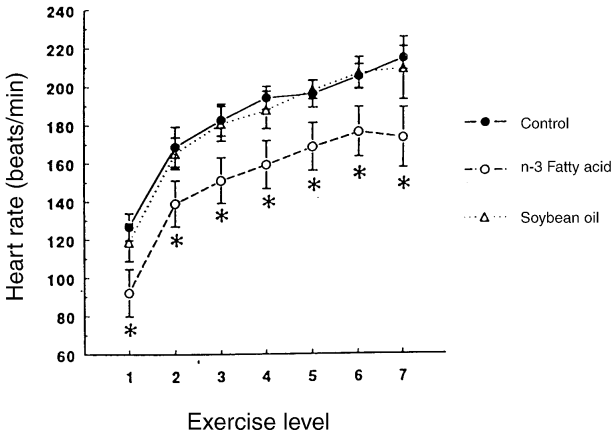

FIG. 2. The heart rate response to submaximal exercise with and without the prior infusion of an emulsion of lipid. These data are from the five animals that received an infusion of n-3 PUFA and a similar infusion of soybean oil (Intralipid®) as a second control. In the remaining eight animals a similar response was observed when normal saline infusion was used as the control. Asterisk indicates control vs. n-3 PUFA, $P < 0.01$, two-factor analysis of variance with repeated measures. For abbreviation and manufacturer see Figure 1.

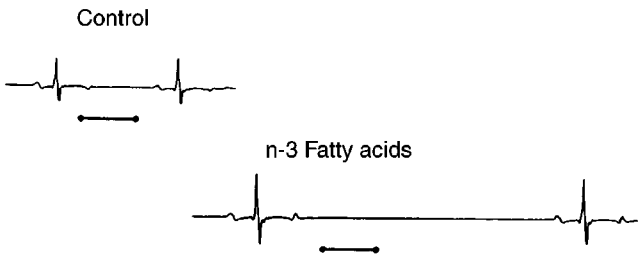

FIG. 3. In a representative portion of an ECG tracing is shown effects of changes in heart rate on the PR and OT intervals produced by the PUFA. Both the control strip and the strip following infusion of the n-3 PUFA were taken with the dog in the resting state. The time bars for both strips indicate 250 ms. The control pulse rate was 107 beats/min, and the n-3 PUFA slowed the pulse rate to 40 beats/min, a larger than average slowing effect in this dog. The PR-interval increased from 100 ms to 120 ms, and most of the prolongation occurred during the isoelectric portion of the interval. The effect of the changes in pulse rate on the QT interval caused by the PUFA can also be demonstrated in these strips. The uncorrected QT interval was measured as 150 ms before the infusion of the PUFA and 170 ms after the infusion. But the QT interval is inversely related to the pulse rate: Slowing the pulse increases the QT interval and vice versa; thus the Bazett's correction (12) is classically made ($QTc = QT/(R–R)^{1/2}$, where QTc is the QT interval corrected for the pulse rate and R-R is the interval between one R-wave and the next one expressed in seconds). Accordingly, QTc is 200 ms before and was reduced to 140 ms after the infusion of the PUFA. See Figure 1 for abbreviations.

these changes in the whole animal relate to specific effects of these PUFA that we have reported in isolated cardiomyocytes, as will be discussed.

Finally, plasma n-3 fatty acids in the nonesterified fatty acid (NEFA), triglyceride, and phospholipid fractions of the serum were analyzed by gas chromatography. After infusion of the fatty acid emulsion, the content of free n-3 fatty acids rose considerably in the NEFA fraction of plasma, as expected (Table 2). However, during the approximate 90 min from the start of the infusion of the emulsion to the end of the study, only minimal EPA and DHA were incorporated into plasma triglycerides with no detectable enrichment in plasma phospholipids. One sees in Table 2 that in all four of the dietary fatty acids of interest which were contained in the egg yolk lecithin (neither linolenic acid LNA nor EPA was detected) there appeared actually to be a decline in their concentration in plasma phospholipids after administration of n-3 PUFA. This unexpected finding might have occurred if we had overcorrected for the possible contribution of the fatty acids in the added egg yolk lecithin. Therefore, in Table 3 we show that, even if no subtraction is made from the total analyzed plasma phospholipids for possible addition of these fatty acids from the egg yolk emulsificant, there occurred no detectable increase in the phospholipids by the time the expected cardiac arrhythmias would have occurred. Incorporation of the PUFA into phospholipids by the liver is rapid and expected to precede incorporation into membrane phospholipids. These PUFA, when incorporated in triglycerides or membrane phospholipids, have not been found to be promptly antiarrhythmic (11,13). With these considerations, our *in vitro* studies are supported that it is only the free form of these fatty acids in the NEFA of the plasma which is responsible for preventing the arrhythmias (13). Indeed, in a single experiment, the infusion of the same PUFA as the ethyl ester failed to prevent arrhythmias during ischemia (data not shown). To minimize use of dogs in these costly experiments, and since similar negative results with the triglyceride and the ethyl ester were obtained *in vitro* (13), further negative studies were not pursued in the dog.

DISCUSSION

The present study demonstrates that the long-chain n-3 PUFA contained primarily in fish oils in contemporary diets can prevent ischemia-induced fatal ventricular arrhythmias in dogs surgically prepared to be susceptible to cardiac sudden death. Thus, we report a statistically significant prevention of ischemia-induced VT or VF in conscious, exercising dogs by the acute infusion of n-3 fatty acids. We further demonstrate the functional effects in these dogs, which are similar to effects we have reported in studies with isolated cardiac myocytes.

Prior *in vitro* studies demonstrated that all long-chain PUFA of the two classes of essential fatty acids, both n-6

TABLE 2
Changes in Fatty Acid (FA) Composition of Six Dietary PUFA in Plasma Lipid Fractions with Infusion of n-3 PUFA[a]

	Plasma lipid fraction											
	NEFA				Triglycerides				Phospholipids			
FA	Control (mM)	After (mM)	Δ (mM)	*P*	Control (mM)	After (mM)	Δ (mM)	*P*	Control (mM)	After (mM)	Δ (mM)	*P*
LA (18:2n-6)	0.063	0.043	–0.020	<0.05	0.039	0.037	–0.002	>0.7	0.578	0.263	–0.315	<0.01
AA (20:4n-6)	0.002	0.020	0.018	<0.01	0.027	0.026	–0.002	>0.05	0.866	0.788	–0.078	>0.1
LNA (18:3n-3)	0	0.004	0.004	>0.2	0	0	0		0	0	0	
EPA (20.5n-3)	0	0.0172	0.017	<0.01	0	0.012	0.012	>0.05	0.014	0.0146	0.0006	>0.7
DPA (22:5n-3)	0	0.019	0.019	<0.01	0	0.006	0.007	>0.3	0.072	0.056	–0.0158	<0.05
DHA (22:6n-3)	0	0.119	0.119	<0.01	0	0.004	0.004	>0.1	0.017	0.002	–0.0172	<0.01

[a]FA compositions were determined before (Control) and after infusion of n-3 PUFA. Values are the means ± SEM. $n = 7$ dogs. Blood samples were obtained prior to starting the n-3 PUFA infusion (Control) and after the infusion but just prior to the exercise–ischemia test (After). Each animal served as its own control. with one control test a week prior to the n-3 PUFA infusion and another a week following the n-3 PUFA infusion. The dose of n-3 PUFA varied from 1.0 to 5 g: the three failures were not dose-related and all occurred with the 5-g dose. They are included in the seven analyses. Since the pure egg yolk lecithin included four of the six FA included in this table (no LNA or EPA were detected), the concentrations of the phospholipid FA were "corrected" in Table 2 by subtracting the estimated concentration of the respective FA contributed from the egg yolk lecithin from the total concentration of these four FA in the plasma phospholipids. LA, linoleic acid; AA, arachidonic acid; LNA, linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; NEFA, nonesterified fatty acids. See Table 1 for other abbreviation.

TABLE 3
Mean of the Paired Differences, Δ*x*, Between the Concentration of EPA and of DHA in Plasma Triglycerides and Phospholipids Following the Infusion of the Fish Oil Emulsion[a]

	(Fish oil) – (Control) Δ*x* ± SEM (mM)	*P*
Triglycerides[b]		
EPA	0.022 ± 0.006	<0.005
DHA	0.0084 ± 0.0034	<0.025
Phospholipids		
EPA	0.0013 ± 0.001	0.2
DHA	0.0034 ± 0.0019	0.1

[a]Infusion of the n-3 PUFA emulsion produced a small incorporation of EPA and DHA in plasma triglycerides, but no detectable incorporation of the EPA or DHA into plasma phospholipids at the time ischemia was induced. In this Table the changes in the phospholipid values found on analysis of the phospholipid fraction after the n-3 PUFA emulsion was infused are uncorrected and therefore include both the content of these four FA in the plasma plus that contributed from the egg yolk lecithin. Nevertheless, as the analysis shows, there is still no detectable increase in the phospholipid content of these FA. See Tables 1 and 2 for abbreviations.

PUFA (abundant in most vegetable oils) and n-3 PUFA (abundant largely in marine foods but present in a few plant oils as well—canola, soybean, flaxseed oils) possess antiarrhythmic actions (13,14). This effect is not shared by the monounsaturated fatty acid, oleic acid, nor by saturated fatty acids. Because of the anomalous behavior of *cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid, 20:4n-6), which in a portion of our experiments produced arrhythmias that can be blocked by cyclooxygenase inhibitors (13), we have recommended that, for potential future clinical applications, studies should focus on the antiarrhythmic actions of the n-3 PUFA, which we have not observed to be proarrhythmic. Therefore, in this study we have examined only the n-3 PUFA of a fish oil concentrate to save on the number of animals needed and to contain the expense of the study.

Free fatty acids are rapidly incorporated by esterification into both plasma triglycerides and phospholipids. The finding in this study, that the prevention of lethal, ischemia-induced ventricular arrhythmias occurred before there was time for any significant incorporation of the n-3 PUFA into plasma phospholipids and minimal incorporation even into triglycerides, suggests that it was the infused free fatty acid form of the PUFA that was antiarrhythmic. These findings confirm our earlier report in isolated myoctes that only the free PUFA, by partitioning into the phospholipid membranes, are necessary to prevent tachyarrhythmias induced by a variety of arrhythmogenic agents (11,13).

Thus these data support the hypothesis that the action of the free, nonesterified PUFA are sufficient for the prompt prevention of ischemia-induced VF in the whole animal, and this can be accomplished by their intravenous administration. Since feeding studies may be confounded by unsuspected variables, we have tested the effectiveness of the n-3 PUFA when administered intravenously as an emulsion of the free fatty acids just prior to inducing myocardial ischemia in the dogs. We have used this protocol to ensure that, if an intravenous emulsion largely of free EPA and DHA prevents arrhythmias, we can with considerable certainty ascribe the protection to an action of the free n-3 PUFA.

When these PUFA are ingested in their natural triglyceride or synthetic ethyl ester form, the intestinal and plasma esterases liberate the free fatty acids and monoglycerides in the intestines. But with absorption they pass through the liver where the free fatty acids are efficiently resynthesized into triglycerides so that the free fatty acid in the NEFA fraction of the plasma lipids is not increased. In the tissues lipoprotein lipases liberate the fatty acids from the transported triglycerides, and the free fatty acids are then carried in the plasma noncovalently bound largely to serum albumin from which they are rapidly transferred to cells. The rate of transfer of bound free fatty acids on albumin is indeed rapid. The time it takes for the fatty acids to leave the albumin in the proximity of cells and to flip-flop across the phospholipid bilayer has been measured and found to be 23 ms or less (15) and is not rate-limiting for the transfer of fatty acids to cell membranes (16), from which the fatty acids can be internalized and be metabolized or incorporated in triglycerides or phospholipids. From the latter stores, the free fatty acid can be liberated by lipases, and it is these liberated free fatty acids which apparently provide the antiarrhythmic effects following stresses which activate the appropriate lipases. With ischemia and myocardial infarction, the catecholamine response will activate lipase activity. If n-3 PUFA are stored in triglycerides or phospholipids as a result of dietary ingestion, they may be available locally at such times to provide protection of the ischemic myocardium from arrhythmias. It has been shown repeatedly that the concentration of free fatty acids rises very promptly in the heart following experimentally induced coronary artery occlusion, though their actual concentration at their site of action on the cardiomyocytes is not known. When we infuse an emulsion of free fatty acids, the aggregates of micellar or laminar forms presumably would deliver their content of free fatty acids directly to cells, bypassing the albumin step to provide the antiarrhythmic effects in the myocardium to subjects who were deficient in stored n-3 PUFA owing to inadequate dietary intake of these protective fatty acids. At present, the question of what constitutes a physiological protective concentration of the free n-3 PUFA at the site of individual cardiomyocytes in the ischemic heart cannot be answered. nor do we know whether the infused emulsion delivers free fatty acids to partition into the cell membrane phospholipids to modulate ion channels at concentrations similar or grossly in excess to that which can be supplied from endogenous stores. We have reported inhibition of the L-type calcium currents in isolated rat heart cells seen with as little as ~20 nM EPA added to the superfusate (17), which is in the physiological concentration of monomeric free fatty acids in the aqueous phase of plasma reported in humans (18,19), but it is the concentration (electrochemical activity) of free n-3 fatty acids that have partitioned into the phospholipids of myocyte membranes that modulate the ion channels, not their concentration in the aqueous phase of plasma. Clearly more research is needed before understanding of the transport and movement in the environment of aqueous plasma of highly hydrophobic mole-

cules, like these fatty acids, provides a definitive answer to this important question. It seems probable to us that the mechanism of the electrically stabilizing, antiarrhythmic effect of the free PUFA on cardiomyocytes, whether ingested or administered acutely intravenously, is the same, but our studies do not exclude the possibility that other additional protection may not be afforded by the PUFA while still incorporated within the membrane phospholipids (20).

The slowing of the heart rate and the prevention of ischemia-induced ventricular fibrillation by the intravenous infusion of n-3 fatty acids in the conscious, exercising prepared dogs were expected from our results with the simpler, isolated, neonatal rat myocyte preparation. In that preparation, in the absence of neural or humoral factors, the PUFA slow the spontaneous beating rate of the cells (13). But they also block the tachyarrhythmias induced by the β-adrenergic receptor agonist, isoproterenol, and its intracellular second messenger, c-AMP (21). Both effects, however, are secondary to the direct, primary electrophysiologic effects of the PUFA on the heart cells (22), which in turn result from modulating effects of the PUFA on ionic currents through membrane ion channels in cardiac myocytes (17,23). The slowing of the heart rate from these actions could contribute to the antiarrhythmic effects of the PUFA.

In these dog studies the effects of the n-3 PUFA included (Table 1) a significant reduction in left ventricular contractility, d(LVP)/dt and in ventricular systolic pressure. Reduced ventricular dP/dt can be caused by a direct depressing effect on the myocardium, but it can also result from changes in blood pressure and heart rate. These same fatty acids decrease blood pressure (24) owing to their vasodilatory actions (25,26) lowering systemic vascular resistance. Since the measurements shown in Table 1 were made before and following administration of the PUFA emulsions, but prior to running on the treadmill, the dogs' heart rates were generally reduced. Ventricular dp/dt is also known to be altered by load conditions both preload and afterload. Therefore, reduction of afterload in this study resulting from the systemic vasodilatation caused by the n-3 PUFA could have contributed to reduction of dP/dt. Although the reduction in inotropy observed in these whole animal studies is significant, it cannot be solely ascribed, from the data available, as resulting from a direct depressing effect on the myocardium.

The antiarrhythmic action of these fatty acids indicates that in the fish oil feeding studies in animals (4–6,20,27) and the one fish and one n-3 fatty acid secondary, randomized clinical trials in humans (28,29) the significant reduction in mortality from sudden death can probably be attributed to the ingestion of these fatty acids. We cannot, however, yet claim that the mechanism for the antiarrhythmic effect is the same when the fatty acids are administered by these different routes, though this seems probable based on the specificity of their antiarrhythmic action (13,17,22,23,30).

The slowing of the heart rate and the reduced QT interval are changes associated with Class Ib antiarrhythmic drugs which prolong the duration of the inactivated state of the voltage-gated sodium channels (31), an effect of the PUFA observed in the isolated myocytes (23). The prolongation of the P-R interval without a change in the P-wave indicates slowing of conduction through the atrio-ventricular node consistent with the inhibition of L-type calcium channels (17) or a β-blocking effect (21) by the PUFA, demonstrated in isolated cardiomyocytes.

The dose of the free acidic PUFA administered in these studies ranged from an initial high dose of 10 g, delivered as a phospholipid emulsion, progressively down to 1 0 g, administered on an albumin carrier to only one animal in this series. Of the three animals not protected from VF by the PUFA, all occurred in dogs that had received 5 g of the n-3 PUFA. Since the other animals were protected by doses that varied from 10 g down to 1.0 g, there was no apparent relation in the 13 animals of this study between the dose and the failure rate.

The free fatty acids cannot be administered intravenously with impunity. The first two animals that received the 100 mL of 10% emulsion rapidly, almost as a bolus injection, died suddenly before the study started. Before the rate of infusion was sufficiently decreased, death occurred several hours later with this high dose in a dog from what appeared to be acute respiratory distress associated with marked hemolysis resulting from the detergent action of the free fatty acids on blood cells. With a slower rate of administration (50 to 60 min) there were no more deaths from the administered PUFA, but hemolysis was visible in the serum and the animals had hematuria on the full 10-g doses. Decreasing the total dose progressively to 7.5, 5, 2.5, and 1.0 g of the emulsion reduced the hemolysis and the hematuria but still afforded protection from fatal arrhythmias. The minimal effective intravenous dose is yet to be determined, but in practice for primary or secondary prevention the oral route will be preferred, though the intravenous use may have application in some acute cardiac emergencies.

In conclusion, the acute intravenous administration of free n-3 PUFA prevents ischemia-induced lethal ventricular arrhythmias in this canine model of sudden cardiac death, and several features of cardiac function induced by the infused n-3 PUFA were similar to the effects of these PUFA in the *in vitro* cardiomyocyte preparation. It is apparent that there is an important regulation of cardiac function based on simple dietary choices of ingested fatty acids, which merits further investigation. Our electrophysiologic studies suggest nervous and other excitable tissues should also be affected by these fatty acids, and this has been shown by others (32–36) and by us (37). There is need now for careful clinical trials by both the oral and parenteral route to determine the efficacy and the optimal safe dose of these n-3 fatty acids in preventing malignant arrhythmias in subjects with/or at risk from cardiovascular diseases—a major public health problem.

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Biliary Excretion of Dolichols and β-Hexosaminidase—Effect of Ethanol and Glucagon

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ABSTRACT: Alcohol has been reported to increase the urinary excretion of dolichols, and urinary dolichols are suggested to be derived from the lysosomes of the renal cells. In the present study we examined the effects of alcohol and glucagon on the biliary excretion of dolichols in rats. Chronic ethanol treatment decreased both biliary dolichol and β-hexosaminidase excretion. The absolute amount of dolichol excreted into the bile correlated highly significantly with the absolute amount of biliary β-hexosaminidase. Our results indicate that biliary dolichols are—at least in part—derived from hepatic lysosomes. Decreased biliary dolichol output during chronic alcohol administration suggests that urinary and biliary dolichol excretions are regulated independently of each other.

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Dolichols are long-chain polyisoprenoid alcohols generally restricted to chain lengths of 14–24 isoprene units (1). They are synthesized in microsomes and stored in lysosomes (1). Dolichols are widely distributed among the tissues of a variety of eucaryotes, in which they occur as free alcohols, esters with long-chain fatty acids, and phosphomonoesters (2,3). The phosphorylated form of dolichol acts as an essential intermediate in the biosynthesis of asparagine-linked glycoproteins (4,5). The free and esterified dolichols have been suggested to play a role in the regulation of membrane fluidity, stability, and permeability (6–11).

Urinary dolichol excretion is increased in a variety of clinical conditions. These include pregnancy (12), severe bacterial infections (12), and malignancies (12,13). Aspirin has been shown to increase the urinary excretion of dolichol and lysosomal enzymes (β-hexosaminidase and β-glucuronidase), which suggests that urinary dolichols may be derived from the lysosomes of the renal cells (14). In addition, alcoholics have elevated urinary (15,16) and blood (17) dolichol levels. Glucagon—a substance known to acutely modify hepatocyte lysosomal function—has been shown to decrease the biliary output of lysosomal enzymes (18). The effect of alcohol on biliary excretion of dolichols has so far not been investigated, although biliary excretion has been considered to be an important elimination route for dolichols (19). Acute ethanol administration has been shown to decrease biliary lysosomal enzyme output in liver perfusate (20). Chronic ethanol feeding, however, has been shown to have no effect on biliary cholesterol or phospholipid output (21).

The aim of the present study was to examine possible mechanisms of biliary dolichol excretion by comparing it to the biliary output of β-hexosaminidase under various *in vivo* conditions.

MATERIALS AND METHODS

General experimental procedure. Male Wistar rats weighing 265–620 g were used for all experiments. Under sodium pentobarbital anesthesia (40 mg/kg), the common bile duct was cannulated just below the hepatic duct bifurcation above the entrance of the pancreatic duct with PE-10 polyethylene tubing. Bile was first collected for 1.5 h. This was followed by the administration of ethanol or glucagon (or an equal amount of saline for control rats) whereafter bile was collected for another 1.5 h. Ethanol [2 g/kg body weight (b.w.)] was administered intraduodenally as a 30% solution whereas glucagon (Novo Nordisk A/S, Bagsvaard, Denmark) was injected intravenously (1 mg/kg b.w.) into the outer jugular vein.

After the total collection period of 3 h, all rats were sacrificed and their livers were removed and weighed. The volumes of collected bile were determined by weighing, assuming a density for bile of 1.0 g/mL.

In the chronic alcohol intake study, a model shown to produce alcoholic liver injury was used (22). After 5 wk of chronic alcohol consumption, the complete bile fistula was created and samples were collected as described above.

Analytical methods. All bile samples were transferred into plastic tubes and kept frozen at −20°C until analyzed. Saponification and extraction of dolichols from bile (0.5 mL) and analysis with a high-performance Varian Vista 5500 liquid chromatograph (Palo Alto, CA) were performed as reported previously (17,23).

β-Hexosaminidase activity (E.C. 3.2.1.30) was determined by a spectrophotometric method using *p*-nitrophenyl-*N*-acetyl-β-glucosamine (Sigma Chemical Co., St. Louis, MO) contained in 0.34 mol/L citrate buffer, pH 4.5 as a substrate (24,25).

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Abbreviation: b.w., body weight.

Statistical calculations. All results are expressed as means ± SEM. Statistical differences between the treatment groups and controls were calculated using Student's *t*-test. Logarithmic transformations were carried out when necessary to ensure that the distributions of the results met the criteria for normality. Spearman's correlation was used in the calculation of correlations. A *P*-value of <0.05 was considered significant.

RESULTS

In all rats, biliary dolichol and β-hexosaminidase excretion tended to decrease during the second collection period. The results of the acute experiments are therefore expressed as the percentage decrease of dolichol or β-hexosaminidase excreted during the second collection period as compared to the first collection period. In the glucagon-treated rats (n = 11), the decrease in biliary dolichol excretion during the second collection period (70 ± 3% decrease of that found in the first collection period) was significantly ($P < 0.01$) larger than that observed in the saline-treated controls (53 ± 5%, n = 10, Fig. 1). The same was also true for β-hexosaminidase (70 ± 5% in glucagon-treated, n = 10, vs. 49 ± 3% in controls, n = 10, $P <$ 0.01, Fig. 1). By contrast, acute ethanol administration had no significant effect on biliary dolichol or β-hexosaminidase excretion during the second collection period as compared to saline-treated controls. Bile flow after acute glucagon administration did not differ significantly from that observed in the controls (0.13 ± 0.02 mL/h/100 g b.w., n = 13, vs. 0.14 ± 0.03 mL/h/100 g b.w., n = 13). The same was also true after ethanol administration (0.18 ± 0.02 mL/h/100 g b.w., n = 14, vs. 0.16 ± 0.02 mL/h/100 g b.w., n = 13).

Chronic alcohol administration led to a decrease in biliary dolichol excretion as compared to controls, and the same was also true for β-hexosaminidase (Table 1). On the other hand, liver weight and bile flow were not different in ethanol-treated and control rats (Table 1).

Combining all experiments for analysis revealed that the absolute amount of dolichol excreted into the bile during a given collection period correlated highly significantly (r = 0.84, $P < 0.0001$) with the absolute amount of β-hexosaminidase excreted (Fig. 2). Similarly, the change observed in biliary dolichol excretion during the first and second collection periods in the acute experiments correlated significantly (r = 0.64, $P < 0.0001$) with the change observed in β-hexosaminidase excretion.

DISCUSSION

Liver plays a central role in the metabolism of dolichols. Radioactively labeled exogenous dolichol is taken up by the hepatocytes and is excreted into the bile in the perfused rat liver (26). Dolichol synthesized by the liver is mainly stored in the

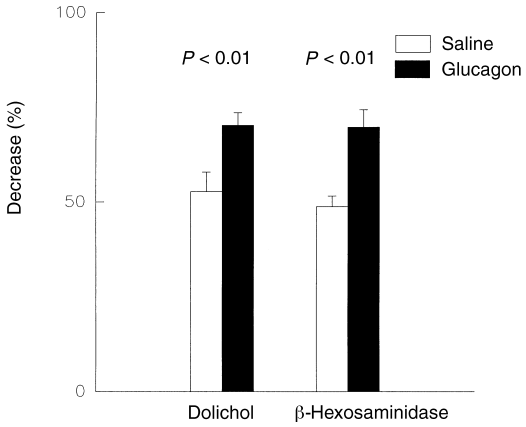

FIG. 1. The percentage decrease (mean ± SEM) in biliary dolichol and β-hexosaminidase excretion after intravenous administration of saline or glucagon to male Wistar rats.

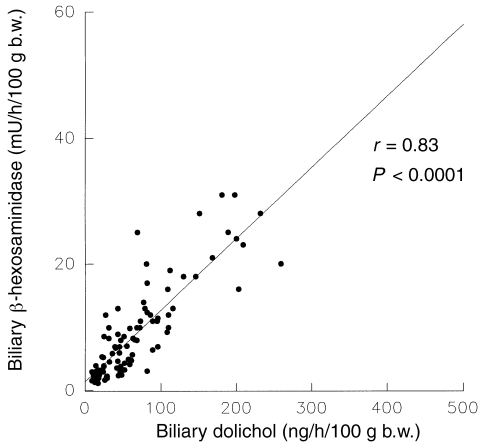

FIG. 2. The correlation between the absolute amount of biliary dolichol and β-hexosaminidase excreted into the bile during the first and second collection period. Abbreviation: b.w., body weight.

TABLE 1
The Effect of Chronic Alcohol Treatment (5 wk) on Biliary Dolichol and β-Hexosaminidase Excretion (mean ± SEM) in Rats[a]

	Dolichol (ng/h 100 g b.w.)	β-Hexosaminidase (mU/h 100 g b.w.)	Bile flow (mL/h 100 g b.w.)	Liver weight (g)
Alcohol (n = 6)	40 ± 8	5.7 ± 1.4	0.18 ± 0.01	12.8 ± 0.7
Control (n = 6)	93 ± 26	11 ± 3	0.22 ± 0.02	12.6 ± 1.2
P-value	0.001	0.001	n.s.	n.s.

[a]Abbreviations: b.w., body weight; n.s., not significantly different.

lysosomes (27). Bile is the only known route for the excretion of substantial amounts of dolichols from the body (19). Mechanisms that regulate biliary dolichol output are largely unknown. Lysosomes constitute the compartment which contains the highest concentration of dolichols in the hepatocytes, and it has been speculated that vesicular transport from lysosomes is involved in the biliary excretion of dolichols (28). Our results confirm that significant amounts of dolichols are excreted into the bile in rats *in vivo*.

Urinary excretion of dolichols is increased after aspirin administration, and this is paralleled by an increased urinary output of two lysosomal enzymes (β-hexosaminidase and β-glucuronidase), which suggests that urinary dolichols are derived from the lysosomes of the renal cells (14). It has been proposed that there also exists a lysosome-to-bile hepatic excretory pathway by which endogenous and exogenous material within the hepatocyte lysosomes may undergo biliary excretion (29). There is also evidence that lysosomes play a role in biliary iron (30) and copper (31,32) excretion.

Glucagon decreased biliary excretion of dolichols and β-hexosaminidase in our experiments. This is in line with previous experiments in which it was shown that glucagon decreases the biliary excretion of lysosomal enzymes in rats (18). The absolute amount of biliary dolichol excretion correlated highly significantly with that of β-hexosaminidase excretion during a given collection period. Similarly, the change observed in dolichol excretion during the first and second collection periods correlated significantly with the change observed in biliary β-hexosaminidase excretion in the acute experiments. The results indicate thus that biliary dolichols are —at least in part—derived from hepatic lysosomes and that biliary excretion of dolichols and lysosomal enzymes may be regulated partly by the same mechanisms.

Chronic ethanol administration decreased the biliary excretion of dolichol and β-hexosaminidase in our settings. The mechanism behind this effect is unclear, but alcohol may somehow disturb the function of lysosomes in the hepatocyte. It has been proposed that ethanol may either impair the movement of lysosomes to the bile canalicular membrane or impair the fusion of lysosomes with the canalicular membrane (20).

The level of dolichol in blood is rather constant under normal conditions (33), and there is no diurnal variation (23). Blood dolichol levels have been reported to be normal in most common diseases as well as in pregnancy (23,33). By contrast, serum dolichol concentration is reported to be highly elevated in two rare lysosomal storage diseases, aspartylglucosaminuria and mannosidosis (34), and to a lesser extent in alcoholics (17). On the other hand, patients suffering from neuronal ceroid lipofuscinosis have a decreased serum dolichol concentration as compared to normal controls (35). The mechanism behind these changes in blood dolichol concentration has been suggested to be a disturbance in lysosomal function (34,35). If dolichols are excreted into the bile by a suggested lysosome-to-bile hepatic excretory pathway (26) and biliary excretion is the main elimination route for dolichols from the body, a disturbance in lysosomal function may explain—at least in part—the elevated serum dolichols found in rare lysosomal storage diseases (34) and alcoholics (17).

Although supporting the concept of lysosomal origin of biliary dolichols, our results—especially the decreasing chronic effects of alcohol on biliary dolichol excretion—indicate that urinary and biliary dolichol excretion are regulated independently of each other or that the kidney is in that respect much more susceptible to the effects of alcohol than the liver.

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Effect of Curcumin and Capsaicin on Arachidonic Acid Metabolism and Lysosomal Enzyme Secretion by Rat Peritoneal Macrophages

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ABSTRACT: The inflammatory mediators secreted by macrophages play an important role in autoimmune diseases. Spice components, such as curcumin from turmeric and capsaicin from red pepper, are shown to exhibit antiinflammatory properties. The influence of these spice components on arachidonic acid metabolism and secretion of lysosomal enzymes by macrophages was investigated. Rat peritoneal macrophages preincubated with 10 μM curcumin or capsaicin for 1 h inhibited the incorporation of arachidonic acid into membrane lipids by 82 and 76%: prostaglandin E_2 by 45 and 48%; leukotriene B_4 by 61 and 46%, and leukotriene C_4 by 34 and 48%, respectively, but did not affect the release of arachidonic acid from macrophages stimulated by phorbol myristate acetate. However, the secretion of 6-keto PG $F_{1\alpha}$ was enhanced by 40 and 29% from macrophages preincubated with 10 μM curcumin or capsaicin, respectively, as compared to those produced by control cells. Curcumin and capsaicin also inhibited the secretion of collagenase, elastase, and hyaluronidase to the maximum extent of 57, 61, 66%, and 46, 69, 67%, respectively. These results demonstrated that curcumin and capsaicin can control the release of inflammatory mediators such as eicosanoids and hydrolytic enzymes secreted by macrophages and thereby may exhibit antiinflammatory properties.

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Inflammation is a necessary response of the host to counteract the threat of infectious agents and other foreign bodies (1). The inflammatory response is coordinated by the number of immune cells such as macrophages, B and T lymphocytes, basophils, eosinophils, and mast cells. A large number of mediators produced by these cells play a key role in inflammatory response. These include arachidonic acid metabolites like prostaglandins (PG) and leukotrienes (LT), reactive oxygen species like superoxide anions and hydroxyl radicals, hydrolytic enzymes like proteases and glycosidases, cytokines, complement-derived peptides, and kinins (2–4). These mediators act in concert to eliminate the source of infection. However if these mediators are produced in an uncontrolled manner, they can lead to the destruction of connective tissue matrix as has been observed in autoimmune diseases like rheumatoid arthritis (5). Enzymes such as collagenase, elastase, and hyaluronidase play a key role in the destruction of cartilage in the joints in arthritic conditions (6). The inflammatory responses can also be exacerbated by cytokines, reactive oxygen species, and arachidonic acid metabolites (4,7). Macrophages present at the site of inflammation are an important source of these various mediators (5). The tissue macrophages at the site of inflammation are supplemented by blood-derived monocytes and lymphocytes which infiltrate the vasculature in large numbers in response to complement activation products, connective tissue fragments, and other endogenously derived stimuli (4,8). Binding of the stimuli to cells leads to transmembrane signaling which ultimately leads to the secretion of lysosomal enzymes (9). To restrict the uncontrolled response of accumulated cells, treatments such as lymphocytopheresis, nonsteroidal antiinflammatory drugs, gold compounds, D-penicillamine, hydroxychloroquinine, corticosteroids, and immunosuppressants are often used (6). These treatments reduce the secretory functions of macrophages and other cells, resulting in the lowering of inflammatory responses.

Although usage of antiinflammatory drugs is in vogue, the continued administration of these drugs over a long period of time can have adverse side effects (5,6,10). Therefore, there is a need to explore alternative strategies to lower the formation of inflammatory mediators with the help of nontoxic natural products. Recently we have demonstrated that phenolic compounds, such as curcumin from the spice turmeric and capsaicin from red pepper, exhibit antiinflammatory properties on carrageenan-induced paw inflammation as well as in adjuvant-induced arthritis in rats (11,12). These effects are comparble to some of the known antiinflammatory drugs such as aspirin, indomethacin, piroxicam, and phenylbutazone (12,13). However, the mechanism by which curcumin and capsaicin exhibit antiinflammatory properties is not very clearly understood yet.

Activation of mononuclear cells plays a very important role in inflammation (5). Since our earlier studies and that of other investigators clearly established the efficacy of cur-

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Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; LT, leukotriene; PG, prostaglandin; PMA, phorbol myristate acetate.

cumin and capsaicin to reduce the inflammation in arthritis and other model systems (11,12,14), it is of interest to study the effect of these phenolic compounds on macrophage activation with reference to secretion of inflammatory mediators. We report in this investigation that curcumin and capsaicin inhibit the incorporation of arachidonic acid into macrophage lipids, which in turn decreases the formation of PGE_2, LTB_4, and LTC_4. Curcumin and capsaicin also transiently reduced the secretion of lysosomal enzymes such as collagenase, elastase, and hyaluronidase in activated macrophages. These studies indicated that the antiinflammatory properties of curcumin and capsaicin may be mediated by their inhibitory effects on the secretion of inflammatory mediators of mononuclear cells.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM), phorbol myristate acetate (PMA), calcium ionophore A23187, Zymosan A, fatty acid-free bovine serum albumin (BSA), indomethacin, *N*-acetyl glucosamine, collagenase (Type VII from *Clostridium histolyticum*), elastase (from bovine pancreas), hyaluronidase (from bovine testes), hyaluronic acid, Dextran T-70, PGE_2, 6-keto prostaglandin $F_{1\alpha}$ and LTB_4 and LTC_4 were obtained from Sigma Chemical Co. (St. Louis, MO). 5,6,8,9,11,12,14,15[^{3}H]Arachidonic acid (specific activity 211.2 Ci/mmol), 6-keto [5,8,9,11,12,14,15(*N*)]-[^{3}H]-$PGF_{1\alpha}$ (specific activity 185 Ci/mmol), and [5,6,8,11,12,14,15(*N*)-[^{3}H]-PGE_2 (specific activity 183 Ci/mmol) were purchased from Amersham International plc (Amersham, United Kingdom). Antisera for 6-keto $PGF_{1\alpha}$ and PGE_2 were purchased from Advanced Magnetics Inc. (Cambridge, MA). DNP-peptide III and Suc-Ala-pNA were bought from Peptide Research Institute (Osaka, Japan). Natural capsaicin was from Aldrich Chemical Co. (Milwaukee, WI). Curcumin (99% pure) was purchased from Flavours and Essences (Mysore, India). Linalool (97% pure) and cuminaldehyde (90% pure) were purchased from Fluka (Buchs, Switzerland). Gelatin was obtained from Difco Laboratories (Detroit, MI). All other chemicals and solvents used were of analytical grade.

Animals. Adult male Wistar rats [OUTB-Wistar, IND-cft (2c), CFTRI animal house facility, Mysore, India] weighing approximately 250 g were used for all the studies.

Isolation of peritoneal macrophages. Macrophages were isolated from the rat peritoneal exudates in Hanks' balanced salt solution (HBSS) as described previously (15).

Uptake of [^{3}H]-arachidonic acid by macrophages. Macrophage monolayers (2.5×10^6 cells) in 1.5 mL of DMEM were incubated with [^{3}H]-arachidonic acid (0.01 μCi complexed to 1 mg BSA/mL) for 15 h at 37°C in a humidified incubator (16). The culture supernatant was aspirated, and the adhering cells were washed three times with DMEM containing 0.1% BSA. The adhering cells were scraped in 1 mL of saline containing 0.01% Triton-X 100, and the lipids were extracted by the method of Bligh and Dyer (17). The lipid extracts were taken in scintillation vials, evaporated to dryness with nitrogen, and dissolved in 5 mL of toluene-based scintillant. The incorporation of [^{3}H]-arachidonic acid was quantitated using a Beckman liquid scintillation counter (Model LS-100; Fullerton, CA).

Release of [^{3}H] arachidonic acid. Macrophage lipids were labeled with [^{3}H]-arachidonic acid as described above and washed three times with DMEM containing 0.1% BSA. The cells were incubated for 2 h in 1.5 mL of DMEM containing 2.5 mg/mL BSA, 3 μM indomethacin, and 0.1 μM PMA (16). Control cells were incubated under similar conditions in the absence of PMA. The radiolabeled arachidonic acid released into the medium was extracted by the Bligh and Dyer procedure (17). The [^{3}H]-arachidonic acid released was quantitated in a Beckman scintillation counter (16).

Release of PG. Macrophage monolayers (2.5×10^6 cells/1.5 mL DMEM) were incubated with 5 μg/mL of Ca^{2+} ionophore A23187 for 90 min. The PGE_2 and 6-keto $PGF_{1\alpha}$ released were extracted from the medium (in 2 mL of ethyl acetate × 3) after acidifying the supernatant with 100 μL of 3% formic acid to pH 3 (18). The combined ethyl acetate extracts were evaporated under a stream of nitrogen and suspended in 100 μL of phosphate-buffered saline containing 1% gelatin. Appropriate dilutions of this solution were used for quantifying the PG by radioimmunoassay (18).

Analysis of LT. Macrophages (2.5×10^6 cells/1.5 mL of DMEM) were activated with 5 μg/mL of Ca^{2+} ionophore A23187 for 90 min. The LT released into the medium were extracted with 2 vol of methanol. The extract was acidified with 3% formic acid to pH 3.0 and prepurified on Sep-Pak C-18 column (Waters, Millipore Corp., Milford, MA) (19). The LT extract was concentrated and loaded on a Supelcosil C-18 high-performance liquid chromatographic column (Supelco, Bellefonte, PA) of pore size of 5 μ. The LT were eluted with acetonitrile/methanol/acetic acid/water (65:10:1:24, by vol) adjusted to pH 5.6 with ammonia. LTC_4 and LTB_4, which eluted at the retention times of 4.7 and 16.9 min, were monitored at 280 nm. The LT were identified and quantitated by comparison with chromatography of authentic standards.

Effect of spice principles on the incorporation and release of arachidonic acid. The macrophages were preincubated with increasing concentration of spice principles (in 10 μL of ethanol) for 60 min prior to the addition of [^{3}H]-arachidonic acid for incorporation studies or prior to the addition of activators in the release reaction. The cells that were incubated with spice principles were thoroughly washed with HBSS containing 2% BSA and used for further studies. Uptake of spice principles by macrophages was monitored by high-performance liquid chromatography analysis as described earlier (15).

Hydrolytic enzymes. Macrophage monolayers (2.5×10^6 cells) were incubated with or without spice principles in 1 mL of HBSS containing 500 μg of Zymosan A for 18–72 h. The hydrolytic enzymes collagenase, elastase and hyaluronidase, released in the culture supernatants by activated macrophages, were measured.

Collagenase activity. Collagenase activity was measured colorimetrically at 365 nm using the synthetic substrate DNP peptide III (DNP-pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg) (20). The amount of DNP peptide hydrolyzed was quantitated using a molar extinction coefficient of 1.49. Collagenase activity is expressed as µM/mg protein (20).

Elastase activity. The activity of elastase was assayed at 405 nm as described by Kawabata *et al.* (21) using the synthetic substrate Suc-Ala-Pro-Ala-pNA in 0.1 M Tris-HCl buffer (pH 5.0). One unit of elastase activity was defined as the quantity of enzyme that liberates 1 µmol of *p*-nitrosanilide in 60 min.

Hyaluronidase activity. Hyaluronidase activity in the culture supernatant was determined by the amount of *N*-acetyl glucosamine released from hyaluronic acid (22,23).

Protein estimation. Total protein was estimated by the method of Sedmak and Grossberg (24) using BSA as reference standard. Macrophage protein was quantified after digesting the cells (2.5×10^6 cells) in 1 mL of 1 N NaOH overnight.

Statistical analysis. The data were statistically analyzed by Student's *t*-test (25).

RESULTS

Effect of spice principles on: (i) Incorporation of arachidonic acid. Macrophages preincubated with curcumin or capsaicin for 1 h incorporated a lesser amount of [^{3}H]-arachidonic acid in membrane lipids as compared to control cells which were incubated with vehicle (10 µL of ethanol) alone. This effect was dependent on the concentration of spice principles used in the incubation medium. Thus, 1, 2.5, 5, 7.5, and 10 µM curcumin inhibited [^{3}H]-arachidonic acid incorporation by 9, 27, 42, 54, and 82%, respectively, at 1 h of incubation (Fig. 1). However, this inhibitory effect of curcumin on fatty acid incorporation was reduced if macrophages were incubated with [^{3}H]-arachidonic acid for a longer period of time. Thus curcumin at 1, 2.5, 5, 7.5, and 10 µM reduced the incorporation of [^{3}H]-arachidonic acid by only 0, 3, 11, 28, and 78% by the end of 2 h. By 6 h of incubation, curcumin at 1 to 5 µM did not inhibit the incorporation of [^{3}H]-arachidonic acid by macrophages. Similarly, the inhibitory effects of curcumin at 10 µM on [^{3}H]-arachidonic acid incorporation were decreased with time, and by 10 h of incubation, curcumin even at high concentrations failed to influence the ability of macrophages to incorporate arachidonic acid (Fig. 1). Capsaicin at 1, 2.5, 5, 7.5, and 10 µM reduced the incorporation of [^{3}H]-arachidonic acid into macrophage lipids by 21, 46, 52, 66, and 76% at 1 h, 3, 11, 18, 36, and 45% at 2 h, 0, 0, 0, 20, and 35% at 6 h, 0, 0, 0, 10, and 27% at 8 h, and there was no reduction at 10 h of incubation. These studies indicated that the inhibitory effect of curcumin and capsaicin on the incorporation of arachidonic acid by macrophages is short-lived and is fully reversible with time. Therefore, the incorporation of arachidonic acid into macrophage lipids was inhibited by spice principles only in the initial period of incubation (<10 h at 10 µM). The macrophages incubated with spice principles were fully viable at all the time points as judged by trypan blue exclusion. Macrophages preincubated with other spice principles such as cuminaldehyde (from cumin) and linalool (from coriander) even at 500 µM had no effect on the incorporation of [^{3}H]-arachidonic acid at all the times tested (Fig. 1). These studies indicate the specificity and the efficacy of individual spice principles in reducing the incorporation of arachidonic acid into macrophage lipids.

The inhibitory pattern of arachidonic acid incorporation into macrophage lipids was associated with intact curcumin or capsaicin inside the cells (Table 1). However, when these spice principles were modified by the cells, the inhibition was reduced. More than 95% of intact curcumin and 87% of capsaicin internalized by the cells are modified by the end of 9 h of incubation (Table 1). However, we were unable to identify the metabolites of curcumin or capsaicin by spectroscopic methods, thin-layer chromatography, or high-performance liquid chromatography. Cuminaldehyde and linalool, though taken up to a maximum extent of 92%, failed to influence the incorporation of arachidonic acid. The cells were fully viable as judged by trypan blue exclusion method at all concentrations of spices used for preincubation.

(ii) Release of arachidonic acid. The macrophages labeled with [^{3}H]-arachidonic acid were preincubated with various spice principles for 1 h. After washing the cells, they were activated with PMA, and arachidonic acid release was quantitated. There was no significant difference in the amount of arachidonic acid released by macrophages preincubated with 1–10 µM curcumin, 1–10 µM capsaicin, 500 µM cuminaldehyde, or 500 µM linalool as compared to that observed with control cells (Table 2). This indicated that spice principles do not affect the phospholipase activity in macrophages. Spice principles alone did not cause the release of arachidonic acid from macrophages. Known inhibitors of phospholipase A_2, like bromophenacyl bromide, inhibited the release of arachidonic acid in a dose-dependent manner (Table 2).

TABLE 1
Internalization of Spice Principles by Macrophages[a]

Time of incubation (h)	Curcumin uptake (µM)	Capsaicin (µM)	Cuminaldehyde (µM)	Linalool (µM)
0.25	0.96	3.29	202	44
0.50	2.75	4.82	300	97
0.75	6.13	7.80	365	239
1.00	9.30	0.68	456	460
2.00	8.50	8.24	426	418
3.00	8.13	8.23	326	307
5.00	3.38	4.10	204	174
7.00	1.75	1.63	89	80
9.00	n.d.	1.24	0.45	22
24.00	n.d.	0.63	0.53	n.d.

[a]Macrophages (2.5×10^6 cells) were incubated in 1 mL of Hanks' balanced salt solution containing curcumin (10 µM), capsaicin (10 µM), cuminaldehyde (500 µM), or linalool (500 µM) in 10 µL ethanol. The spice principles internalized were quantitated by high-performance liquid chromatography (Ref. 15). Values are mean of three independent experiments; n.d., not detected.

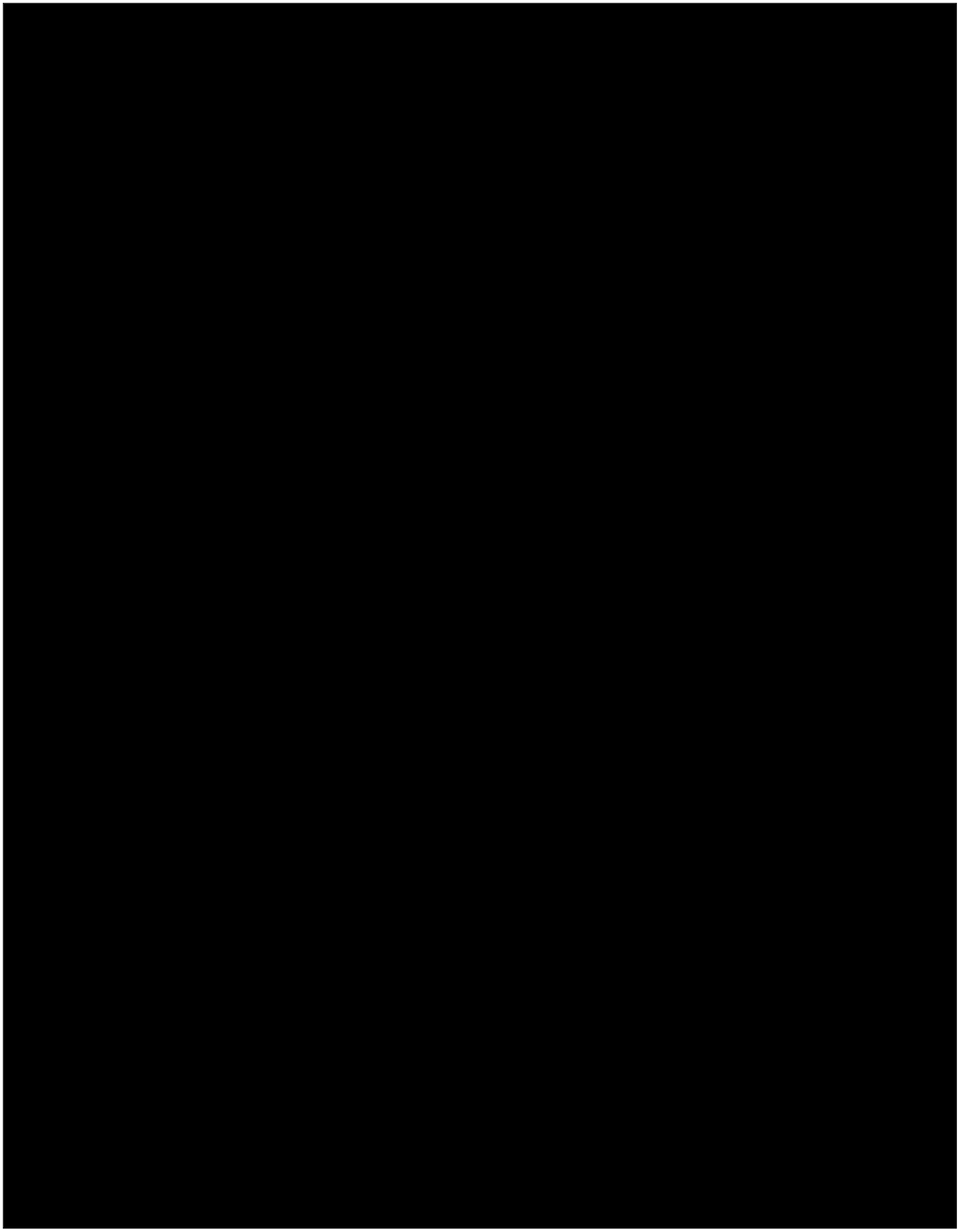

FIG. 1. Effect of spice principles on the incorporation of [^{3}H]-arachidonic acid into macrophage lipids *in vitro*. The incorporation of [^{3}H]-arachidonic acid into macrophage lipids was determined after incubation with (A) curcumin or (B) capsaicin at the following concentrations: A: 0; B: 1 μM; C: 2.5 μM; D: 5 μM; E: 7.5 μM, and F: 10 μM or (C) cuminaldehyde or (D) linalool at 500 μM concentrations. Arachidonic acid incorporation was measured at the time intervals indicated. Values with same superscripts are not significantly different from each other at $P < 0.05$. Abbreviation: NS, not significantly different from the control value.

TABLE 2
Effect of Spice Principles on the Release of [^{3}H]-Arachidonic Acid from Macrophages

Compound added (μM)	[^{3}H]-Arachidonic acid released (cpm/mg protein/h)
Nil	16338 ± 235
Curcumin	
1.0	16637 ± 400
5.0	15987 ± 259
10.0	16365 ± 103
Capsaicin	
1.0	15907 ± 395
5.0	16100 ± 288
10.0	16564 ± 269
Cuminaldehyde (500)	16054 ± 203
Linalool (500)	16299 ± 270
Bromophenacyl bromide	
1.0	7414 ± 410
5.0	4392 ± 186
10.0	2024 ± 398

[a]Macrophages (2.5×10^6 cells/mL Dulbecco's modified Eagle's medium) were preincubated with [^{3}H]-arachidonic acid–bovine serum albumin complex followed by spice principles for 60 min. The release of [^{3}H]-arachidonic acid was measured in the supernatant after activating the cells with phorbol ester as described in the Materials and Methods section. Values are means ± SD, n = six experiments.

(iii) Effect on PG formation. Since arachidonic acid uptake was influenced by curcumin and capsaicin, their effects on arachidonic acid metabolites (PG and LT) were determined. Macrophages preincubated with curcumin or capsaicin secreted lower levels of PGE_2 as compared to that produced by control cells when activated with calcium ionophore (Table 3). Thus, macrophages preincubated with 1 and 10 μM curcumin produced 22% ($P < 0.05$) and 45% ($P < 0.05$) lower amounts of PGE_2, respectively, compared to control cells. Similarly, preincubation of macrophages with 1 and 10 μM capsaicin produced 40% ($P < 0.05$) and 48% ($P < 0.05$) lower amounts of PGE_2, respectively, compared to that produced by control cells.

TABLE 3
Effect of Spice Principles on the Release of Prostaglandins by Macrophages *in vitro*[a]

Spice principle added (μM)	PGE_2 (μg/mg protein)	6-Keto-$PGF_{1\alpha}$ (μg/mg protein)
Nil	49.16^a ± 5.95	58.08^a ± 3.05
Curcumin		
0.1	47.50^a ± 4.93	55.17^a ± 13.82
1.0	38.33^b ± 1.66	$70.b8^b$ ± 6.54
10.0	27.00^c ± 3.32	81.25^c ± 5.84
Capsaicin		
0.1	44.17^a ± 2.76	52.38^a ± 3.05
1.0	29.33^c ± 6.63	67.04^b ± 9.74
10.0	25.34^c ± 3.23	75.10^b ± 13.82
Cuminaldehyde		
500	50.14^a ± 2.93	56.77^a ± 2.19
Linalool		
500	52.49^a ± 4.15	49.56^a ± 3.96

[a]Values with different superscripts in the same column are significantly different from the control values (without spice principles) ($P < 0.05$). Values are means ± SD of three experiments. PGE_2, prostaglandin E_2; $PGF_{1\alpha}$, prostaglandin $F_{1\alpha}$.

However, 6-keto $PGF_{1\alpha}$ release was consistently increased in macrophages incubated with curcumin or capsaicin. Curcumin treatment at 1 and 10 μM enhanced 6-keto $PGF_{1\alpha}$ by 22% ($P < 0.05$) and 40% ($P < 0.05$), respectively, while capsaicin at 1 and 10 μM enhanced 6-keto $PGF_{1\alpha}$ by 15% ($P < 0.1$) and 29% ($P < 0.05$), respectively, compared to control cells (Table 3). Macrophages treated with cuminaldehyde and linalool, even at 500 μM levels, had no influence on secretion of PGE_2 and 6-keto $PGF_{1\alpha}$.

(iv) Effect on LT formation. LT are potent inflammatory lipoxygenase products. Macrophages preincubated with increasing concentrations of curcumin or capsaicin (1–10 μM) secreted lower amounts of LTB_4 and LTC_4 (Fig. 2). The inhibitory effects on LTB_4 and LTC_4 secretion were observed even at low concentrations of curcumin or capsaicin. A maximum inhibition of 61 and 46% LTB_4 formation was observed ($P < 0.05$) when macrophages incubated with 10 μM curcumin or capsaicin, respectively. Similarly, LTC_4 was decreased by 34 and 48% ($P < 0.05$) when macrophages were incubated with 10 μM curcumin or capsaicin, respectively (Fig. 2). Cuminaldehyde and linalool even at 500 μM did not affect LT formation in macrophages (data not shown).

(v) Effect on lysosomal enzymes. Hydrolytic enzymes play an important role in the tissue destruction observed in autoimmune diseases. Macrophages, when treated with curcumin (10 μM) or capsaicin (10 μM), secreted lower levels of lysosomal enzymes like collagenase, elastase, and hyaluronidase (Fig. 3). With curcumin, the amount of collagenase secreted by macrophages activated with Zymosan was reduced by

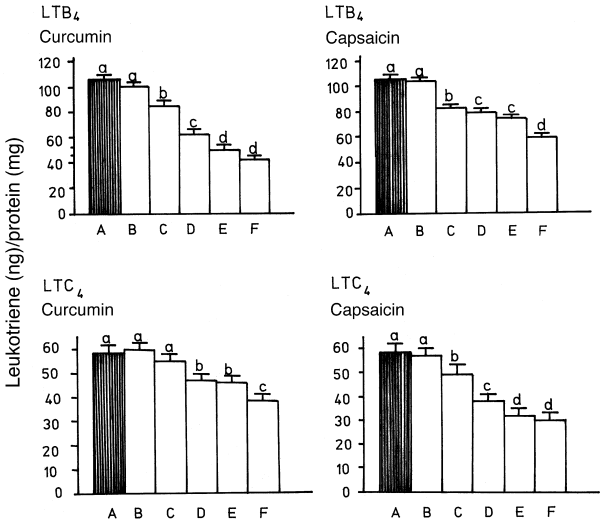

FIG. 2. Effect of spice principles on the release of leukotrienes by macrophages *in vitro*. Leukotrienes B_4 (LTB_4) and C_4 (LTC_4) released by macrophages preincubated with curcumin or capsaicin were quantitated by high-performance liquid chromatography as described in the Materials and Methods section. The concentrations of curcumin or capsaicin added to macrophage monolayers in Dulbecco's modified Eagle's medium (DMEM) were as follows: A: 0, B: 1 μM; C: 2.5 μM; D: 5 μM; E: 7.5 μM; and F: 10 μM. Values are means ± SD of six experiments. Values with the same superscripts are not significantly different from each other at $P < 0.05$.

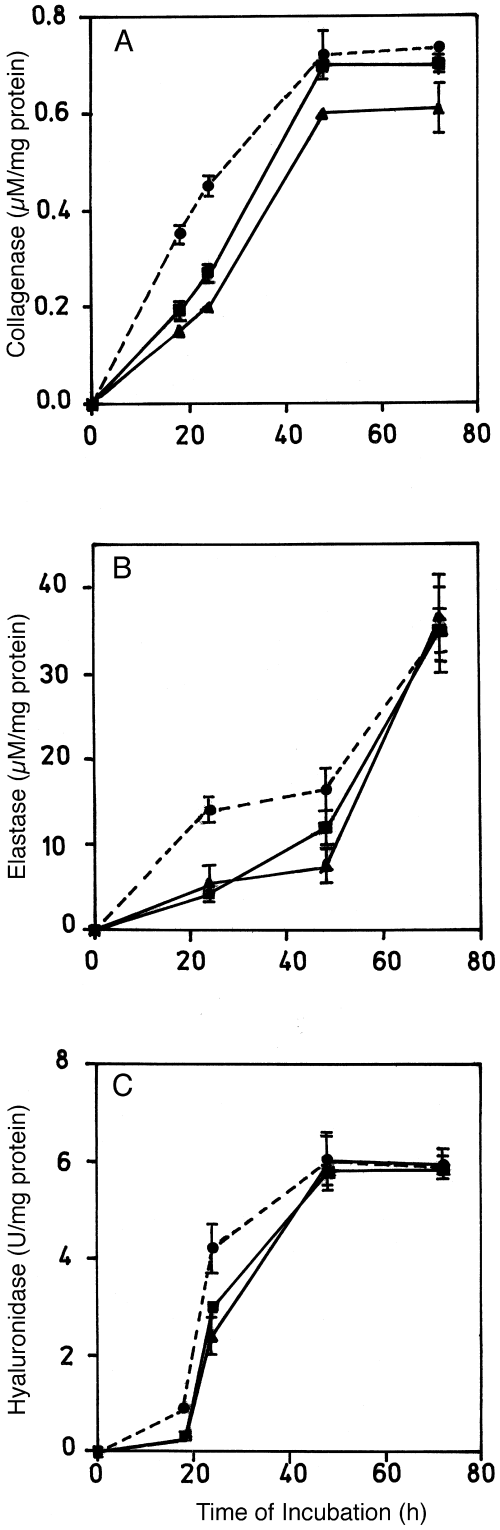

FIG. 3. Effect of spice principles on the secretion of hydrolytic enzymes by macrophages *in vitro*. Macrophages (2.5×10^6 cells/mL DMEM) were incubated with 10 μM curcumin (----) or 10 μM capsaicin (——) along with Zymosan A for different time intervals. The release of hydrolytic enzymes (A) collagenase, (B) elastase, and (C) hyaluronidase was assayed as described in the Materials and Methods section and compared with that of control cells (●---●) incubated under similar conditions in the absence of spice principles. Values are means ± SD of six experiments. See Figure 2 for abbreviation.

57% ($P < 0.001$), 56% ($P < 0.001$), 17% ($P < 0.001$), and 17% ($P < 0.001$) at the end of 18, 24, 48 and 72 h, respectively. Similarly, elastase secretion was reduced in curcumin-treated macrophages by 61% ($P < 0.001$) and 55% ($P < 0.001$), respectively, at the end of 24 and 48 h (Fig. 3). Hyaluronidase secretion was decreased in macrophages incubated with curcumin by 66% ($P < 0.001$) and 43% ($P < 0.001$) at 18 and 24 h, respectively (Fig. 3). The inhibitory effect of curcumin on elastase and hyaluronidase secretion by macrophages was, however, completely overcome when macrophages were incubated for a longer period of time (>72 h). Similarly, capsaicin-inhibited collagenase secretion from macrophages by 46% ($P < 0.001$) and 40% ($P < 0.001$) at the end of 18 and 24 h, respectively; elastase secretion by 69% ($P < 0.001$) and 25% ($P < 0.05$) at 24 and 48 h, respectively; and hyauronidase secretion by 67 ($P < 0.001$) and 30% ($P < 0.05$) at the end of 18 and 24 h of incubation, respectively (Fig. 3). Here again, the inhibitory effects on lysosomal enzyme secretion were not observed when the cells were incubated for longer periods of time with capsaicin (>72 h) (Fig. 3). Spice principles themselves did not affect the activities of collagenase, elastase, and hyaluronidase (data not shown).

DISCUSSION

These studies indicate that macrophage functions are altered by curcumin and capsaicin. Both these phenolic compounds, in a dose-dependent manner, inhibit the incorporation of arachidonic acid into macrophage lipids. However, when macrophages were subsequently activated with PMA after the incorporation of arachidonic acid, no significant differences in the percentage of arachidonic acid released were observed in curcumin- and capsaicin-treated macrophages as compared to that released by control cells. This indicated that curcumin and capsaicin did not affect the release of arachidonic acid from macrophage lipids. The incorporation of arachidonic acid into tissue lipids requires activation of fatty acids by fatty acyl CoA synthetase and transfer by fatty acyl transferase (26). The specific effects of curcumin and capsaicin on these two enzymes have yet to be ascertained. Curcumin is reported to inhibit Δ^6 desaturase in *Mortierella alpina* and rat liver microsomes (27). This lowers the conversion of linoleic to arachidonic acid. As a consequence of these effects on Δ^6 desaturase and fatty acid uptake, curcumin lowers the overall arachidonic acid levels in the tissues. The lower levels of arachidonic acid subsequently reflected in reduced production of PGE_2, LTB_4, and LTC_4, which are major arachidonic acid metabolites formed from cyclooxygenase and lipoxygenase pathways. These results are in agreement with those found in neutrophils (28) and skin fibroblasts (29) where curcumin decreased eicosanoid formation. However, the formation of 6-keto $PGF_{1\alpha}$ was consistently enhanced after the treatment of macrophages with curcumin and capsaicin. This indicated a differential influence of these phenolic compounds on PG and prostacyclin synthetases. It is also possi-

ble that arachidonic acid may be channeled into prostacyclin synthesis by these compounds. Antioxidants such as ascorbate have been shown to enhance the formation of 6-oxo $PGF_{1\alpha}$ in ram seminal vesicles (30). Capsaicin also produces activation of a number of biochemical systems increasing cellular cGMP, diacylglycerol, and inositol triphosphate (31). It is interesting to note that capsaicin and its structural analogs are reported to have vasodilatory effects (32). Prostacyclin is a vasodilatory compound which is enhanced in macrophages by curcumin and capsaicin treatment. Analogs of capsaicin have analgesic and antiinflammatory properties (31). Capsaicin has been used to treat a number of painful clinical conditions such as cluster headache, postmastectomy pain, diabetic neuropathy, and rheumatoid arthritis (33,34). Similarly, antiinflammatory properties of curcumin have also been reported. Srivastava *et al.* (35) have demonstrated that curcumin inhibits platelet aggregation and eicosanoid formation in human blood platelets. Thus, curcumin and capsaicin can alter arachidonic acid metabolism and the generation of other inflammatory mediators.

The mononuclear cells are known to generate enzymes which are potent mediators of inflammation that can destroy tissues (5). These cells may contribute to the excessive levels of collagenase and PG in synovitis (9,36). This, in turn, may contribute to the destruction of connective tissues and the erosion of bone, cartilage, and soft connective tissues. Therefore, controlling the secretion of lysosomal enzymes, such as collagenase, elastase and hyaluronidase, from mononuclear cells may reduce inflammatory responses. Curcumin and capsaicin inhibit the secretion of these various enzymes from macrophages in the early stages of activation. Nirmala and Puvanakrishnan (37) reported that curcumin can lower the elevated levels of lysosomal hydrolases such as β-glucuronidase, β-*N*-acetyl glucoseaminidase, cathepsin B, cathepsin D, and acid phosphatase in myocardial infarction. Similarly, Srivastava and Srimal (34) have shown that curcumin stablizes the lysosomal membranes and inhibits the liver acid phosphatase and cathepsin D activities. The release of collagenase by human leucocytes is enhanced by PGE_2 and cAMP (38). Curcumin and capsaicin inhibited the synthesis of PGE_2. As a consequence of this, the release of collagenase and other lysosomal enzymes may have been decreased in the cells treated with curcumin and capsaicin. Our earlier studies have shown that the formation of reactive oxygen species in macrophages is also inhibited by curcumin and capsaicin (15). Nitric oxide is known to activate metalloprotease enzymes in articular cartilage (39). Reducing nitric oxide by spice principles lowers the activation of proteases and thereby exerts antiinflammatory properties. Therefore, several of the mediators of inflammation secreted by macrophages could be reduced by curcumin and capsaicin.

The effects of curcumin and capsaicin on macrophages, however, are reversible. The inhibitory effects of these compounds on arachidonic acid metabolism are evident only when intact molecules are present in the cells. Macrophages are capable of metabolizing these compounds since the parent molecules were not found in the cells with increasing time of incubation. Our attempts to identify the metabolites of curcumin and capsaicin by conventional methods were not successful. Similarly, Vijayalakshmi and Chandrasekhara (40) earlier demonstrated the rapid turnover of curcumin in rats but were unable to detect the metabolites. Recently Oi *et al.* (41) have shown a capsaicin-degrading enzyme in rat liver microsomes. Here, again, the end product was not identified. However, the effects of curcumin and capsaicin on lysosomal enzymes were observed even after intact spice principles disappeared from macrophages.

The studies with spice components have already established the nontoxic, antimutagenic, antioxidant, antimicrobial, and antiinflammatory effects of curcumin and capsaicin (33,42–44). No adverse side effects have been found with the consumption of curcumin even at a very high dose of 1% in the diet (45). The spices are unlikely to accumulate in the body and cause any adverse side effects due to their rapid turnover. Mega doses of curcumin (1800 to 2500 mg/day) have been successfully used on human arthritic subjects with encouraging results in the alleviation of inflammation (42). Therefore, phenolic compounds from spices, like curcumin and capsaicin, may provide an adjunctive means to reduce the production of inflammatory mediators from mononuclear cells.

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Generation and Remodeling of Highly Polyunsaturated Molecular Species of Rat Hepatocyte Phospholipids

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ABSTRACT: Freshly isolated rat hepatocytes were incubated for 20 min with [U-^{14}C]glycerol in the presence or absence of unlabeled linoleic (18:2n-6), arachidonic (20:4n-6), or docosahexaenoic (22:6n-3) acid, added as albumin complex in 10% ethanol. Most of the radioactivity (~95%) recovered in hepatocyte lipids was present in phosphatidylcholine (PC), phosphatidylethanolamine (PE), and triacylglycerol (TAG). The presence of exogenous fatty acids resulted in (i) higher incorporation of [U-^{14}C]glycerol, (ii) higher percentage of label in TAG, and (iii) enhanced formation of PC and PE molecular species bearing the exogenous fatty acid at both the *sn*-1 and *sn*-2 positions of glycerol. In each case, these molecular species contained 60 to 70% of the label in that lipid class. Further incubation of the cells for 40 and 80 min in the absence of labeled substrate and exogenous fatty acids resulted in a redistribution of label among PC and PE molecular species due to deacylation–reacylation at the *sn*-1 position of glycerol.

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The glycerophospholipids of mammalian cells, including rat hepatocytes, consist of specific molecular species which differ from one another in their relative amounts, physical properties, and half-life times. They most likely fulfill certain structural requirements as membrane components and serve as resources for the generation of signaling molecules (for reviews see Refs. 1–4). All cellular glycerophospholipids are continually synthesized, remodeled, transported between organelles and membranes, and degraded. The regulation of these processes is of particular importance for the major membrane lipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) which, in rat hepatocytes, constitute over 75% of the cell's glycerophospholipids (5).

It has long been known that individual molecular species of rat hepatocyte PC and PE have drastically different half-life times ranging from 1 to 20 h (6,7). Based on both stable isotope and radioisotope-labeling experiments, we have proposed that the molecular species of both PC and PE are generated by continuous *de novo* synthesis and remodeling by deacylation–reacylation at both the *sn*-1 and *sn*-2 positions of glycerol.

Much evidence exists that phospholipid synthesis and turnover in hepatocytes are highly compartmentalized and that interorganelle movement (8,9) as well as lipoprotein assembly and secretion involve newly synthesized phospholipids (10–13). It is therefore logical to assume that remodeling by deacylation–reacylation also involves newly synthesized molecular species of PC and PE (14,15).

Based on acyl turnover measurements by ^{18}O incorporation from $H_2{}^{18}O$-containing media, we have proposed that *de novo* synthesis and remodeling of rat hepatocyte PC and PE are quantitatively similar and linked temporally and spatially. In this scheme, *de novo* synthesis *via* the Kennedy pathway (16) would result in the formation of primarily four molecular species, and the major remodeling step would be the introduction of stearic acid (18:0) and arachidonic acid (20:4) at the *sn*-1 and *sn*-2 positions, respectively, of newly formed PC and PE (5). This hypothesis is in agreement with a great variety of early data on the labeling patterns of PC and PE in liver cells and subcellular preparations (reviewed in Refs. 1,2).

Although stearic and arachidonic acids are primarily introduced into PC and PE by the remodeling pathway, small amounts of 1,2-diarachidonoyl PC exist in many cells (17–19), including rat hepatocytes where they appear to be synthesized *de novo via* glycerophosphate (15). If these and other highly unsaturated molecular species are continually synthesized but remain at low levels, they must be either continually degraded or remodeled into other species. Furthermore, if cells are exposed to excess fatty acids they would be expected to be taken up into glycerolipids by the *de novo* pathway owing to the finite amounts of precursor species available for remodeling. Indeed, we have shown that excess fatty acids, including arachidonic acid, are incorporated into PC primarily by the *de novo* pathway resulting in the initial formation of molecular species having the exogenous fatty acid esterified at both the *sn*-1 and *sn*-2 positions. In the case of 20:4-20:4 PC, rapid remodeling occurred at the *sn*-1 position resulting in the formation of the predominant molecular species 18:0-20:4 PC (15).

The purpose of the present study was to determine if other polyunsaturated fatty acids, when present in excess, are in-

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Abbreviations: DAG, diacylglycerols; DNB, dinitrobenzoate; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerols; TLC, thin-layer chromatography. Also, 18:2, 20:3, 20:4, 22:5, and 22:6 denote 18:2n-6, 20:3n-6, 20:4n-6, 22:5n-3, and 22:6n-3, respectively.

corporated into glycerolipids by analogous pathways and whether newly synthesized polyunsaturated molecular species of PE are remodeled at the *sn*-1 position as previously shown for PC (15).

MATERIALS AND METHODS

Materials. Male Sprague-Dawley rats, 200–350 g, were obtained from Sasco, Inc. (Omaha, NE). Collagenase Type IV from *Clostridium histolyticum*, phospholipase C from *Bacillus cereus*, 4-dimethylaminopyridine, 3,5-dinitrobenzoylchloride, and dilinoleoyl PC were from Sigma (St. Louis, MO). Diarachidonoyl PC was from Serdary Research Labs (Englewood Cliffs, NJ), [U-^{14}C]glycerol (130 Ci/mol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO); unlabeled fatty acids and tridocosahexaenoylglycerol were from Nu-Chek-Prep (Elysian, MN). 1,2-Didocosahexaenoylglycerol was prepared by the Grignard procedure (20,21). Briefly, 10 mg tridocosahexaenoyl glycerol was dissolved in 1.5 mL diethyl ether. Fifty μL of ethyl magnesium bromide (3 M solution in diethyl ether; Aldrich Chemical Co., Milwaukee, WI) was added. After 30 s at room temperature, the reaction was terminated with 50 μL of glacial acetic acid followed by 1 mL of water. 1,2-Diacylglycerols (DAG) in the organic phase were purified by thin-layer chromatography (TLC) on silica gel H, developed in hexane/diethyl ether/glacial acetic acid, (50:50:1, by vol).

Preparation of cells. Hepatocytes were prepared by collagenase perfusion of rat liver according to Krebs *et al.* (22) as described previously (5,14,15). Cells were counted in a hemacytometer after mixing with trypan blue in order to assess viability, which was better than 87% at the beginning and 77–81% at the end of the experiments.

Incubations. Fatty acids were dissolved in 95% ethanol, and 0.1 mL of this solution was added to 0.9 mL of incubation buffer (Krebs-Ringer Hepes containing 10 mM glucose and 0.4% fatty acid-free bovine serum albumin). The mixtures were vortexed and sonicated to obtain a homogeneous suspension. Of these suspensions, 50-μL aliquots were added for each milliliter of incubation buffer. Hepatocytes were incubated at a concentration of 4×10^6 cells/mL of incubation buffer which also contained 1 μCi (7.7 nmol) [U-^{14}C]glycerol/mL and 300 nmol fatty acid/mL. Control incubations contained ethanol only. For each regimen, 2.4×10^7 cells (6 mL) were incubated for 20 min in a 70-mm plastic culture dish, swirled gently in an atmosphere of O_2/CO_2, 95:5, at 37°C. The cells were then washed and resuspended in 6 mL of fresh incubation buffer. Two 1-mL aliquots were removed and extracted immediately, and four 1-mL aliquots were transferred to 30-mm plastic culture dishes and incubated further for 40 and 80 min in the absence of fatty acid and [U-^{14}C]glycerol. Duplicate samples were extracted and analyzed separately. All primary data represent average values from duplicate incubations.

Lipid analysis. Cells were harvested by centrifugation and extracted with chloroform/methanol (2:1, vol/vol) and partitioned against 0.9% aqueous NaCl (23). The lipid extracts were taken to dryness and redissolved in 1 mL of chloroform/methanol, 2:1, containing 0.01% butylated hydroxytoluene. Aliquots of the lipid extracts were fractionated by TLC on Whatman LK5 plates (Whatman, Inc., Clifton, NJ), using chloroform/methanol/ammonium hydroxide/water (65:35:5:1, by vol) as developing solvent. Distribution of radioactivity in lipid classes was determined by scanning the plate with a Berthold LB2842 TLC Linear Analyzer (Berthold Analytical Instruments, Inc., Nashua, NH). Total phospholipids and individual phospholipid classes were quantified by phosphorus assay (24).

Both PC and PE were isolated by preparative TLC on layers of Silica Gel H (Merck, EM Science, Gibbstown, NJ) using the same solvent system. They were checked for purity, hydrolyzed to DAG, and converted to 3,5-dinitrobenzoates (DNB) as described (5,14,15). DAG–DNB were separated on a 3.9×300 mm Waters Nova Pak C18 (Millipore Corp., Milford, MA) column, using a Beckman high-performance liquid chromatography (HPLC) system consisting of two 114 M solvent delivery modules, a Model 406 Analog Interface Module, a Model 168 Diode Array Detector, and System Gold software (Beckman Instruments, Inc., Fullerton, CA). The absorbance at 254 nm was monitored on the computer screen and the fraction collector (Frac-100, Pharmacia Biotech, Inc., Piscataway, NJ) was advanced manually at the beginning of each peak.

Separation and analysis of molecular species as DAG–DNB were done as previously described (5,14,15). Briefly, one-half of each sample was separated by reverse-phase HPLC in a solvent system of methanol/isopropanol/glacial acetic acid (80:20:0.01, by vol), and the peaks were collected in 7-mL scintillation vials. After evaporation of the solvent, 3 mL of Ecolume scintillation fluid (ICN Pharmaceuticals, Costa Mesa, CA) was added, and radioactivity was determined in a Beckman LS7500 scintillation counter. Radioactivity in each fraction was expressed as a percentage of radioactivity recovered in all fractions. The second half of each sample was separated in the same manner, and the peaks were collected for subsequent separation of coeluting species in a solvent of acetonitrile/isopropanol (70:30, vol/vol). Mol% of each molecular species was determined by integration of the HPLC peaks obtained with the two solvent systems. Data from the first and second separations were combined to give radioactivity percentage and mol% of all major molecular species of PC and PE.

RESULTS

Incorporation of [U-^{14}C]glycerol into hepatocyte glycerolipids in the presence and absence of exogenous fatty acids. When primary isolates of rat hepatocytes were incubated for 20 min with [U-^{14}C]glycerol in the presence or absence of unsaturated fatty acids, most of the radioactivity (~95%) was recovered in PC, PE, and triacylglycerol (TAG) (Table 1). Further incubation for 40 and 80 min after removal of labeled substrate led to increased levels of radioactivity in

TABLE 1
Incorporation of [U-^{14}C]Glycerol into Rat Hepatocyte Glycerolipids in the Presence and Absence of Polyunsaturated Fatty Acids[a]

Addition	Time (min)	dpm/μmol lipid P[b] (%) PC[c]	PE[c]	TAG[c]
Ethanol	20	51.5 (22.8)	49.2 (21.7)	125.0 (55.4)
	60	73.3 (26.9)	61.7 (22.6)	137.3 (50.4)
	100	80.0 (25.5)	67.0 (21.3)	166.9 (53.2)
18:2	20	148.1 (14.3)	87.7 (8.4)	802.7 (77.3)
	60	144.3 (14.8)	101.3 (10.4)	728.3 (74.8)
	100	127.8 (13.2)	96.6 (10.0)	741.9 (76.8)
20:4	20	119.6 (13.8)	87.1 (10.1)	654.0 (75.9)
	60	120.4 (13.2)	105.9 (11.6)	682.7 (75.1)
	100	127.8 (13.2)	96.6 (10.0)	742.0 (76.8)
22:6	20	111.7 (18.0)	83.8 (13.5)	423.4 (68.4)
	60	111.4 (15.2)	109.5 (14.9)	511.8 (69.9)
	100	103.3 (14.1)	99.1 (13.6)	526.6 (72.2)

[a]Data are averages of two separate incubations; they do not include small amounts of radioactivity in other lipid classes.
[b]Total phospholipid P recovered in the lipid extract.
[c]Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerols.

control cells. However, no changes in the distribution of radioactivity among these lipid classes occurred, suggesting that there had been some continued synthesis from labeled intracellular precursors. This was not the case with incubations containing exogenous fatty acids. The presence of exogenous fatty acids resulted in increased overall [U-^{14}C]glycerol incorporation and a higher percentage of radioactivity in TAG. Although there was some variability in the levels of radioactivity, the distribution of label among these three lipid classes remained approximately the same for each experiment after withdrawal of the labeled substrate.

Generation of highly polyunsaturated molecular species. Both PC and PE were isolated by preparative TLC, hydrolyzed by phospholipase C, and the resulting DAG were converted to their DNB. Reverse-phase HPLC of DAG–DNB followed by recovery and refractionation of unresolved mixtures resulted in the separation and analysis of individual molecular species. They were identified by the use of synthetic standards and gas chromatographic analysis of their constituent fatty acids, essentially as described previously (15).

As shown in Figure 1, the presence of exogenous fatty acids in the incubation medium resulted in the generation of molecular species having these fatty acids esterified at both the *sn*-1 and *sn*-2 positions of glycerol. After 20 min of incubation in the presence of 18:2, the 18:2-18:2 PC increased from about 1 mol% of total molecular species to 2.3%. In the presence of 20:4, the fraction containing the 20:4-20:4 species increased from 0.3 to 1.8%, and incubation with 22:6 resulted in the appearance of a 22:6-22:6 species (0.7%) that was not detectable (<0.1%) in the control experiment. These data demonstrate that rat hepatocytes can utilize exogenous polyunsaturated fatty acids for the synthesis of highly unsaturated glycerolipid molecular species, and the major newly synthesized species have the exogenous fatty acid at both the *sn*-1 and *sn*-2 positions of glycerol.

Further incubation of hepatocytes for 40 and 80 min in the absence of exogenous fatty acids reduced the amounts of

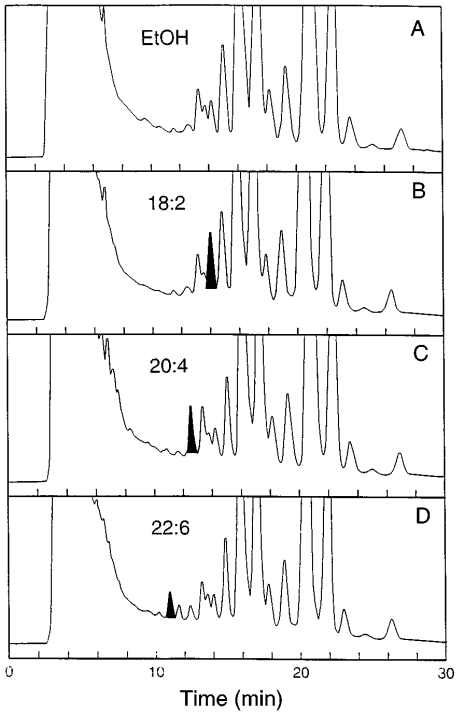

FIG. 1. Fractionation of phosphatidylcholine (PC) molecular species as diacylglycerol dinitrobenzoate (DAG–DNB) derivatives. The tracings represent first-run high-performance liquid chromatography (HPLC) separations of samples obtained from hepatocytes incubated for 20 min with ethanol (A), and 0.3 mM each of linoleic acid (B), arachidonic acid (C), and docosahexaenoic acid (D). Shaded peaks represent molecular species bearing the exogenous fatty acid at both the *sn*-1 and *sn*-2 positions of glycerol. HPLC conditions are described in the Materials and Methods section.

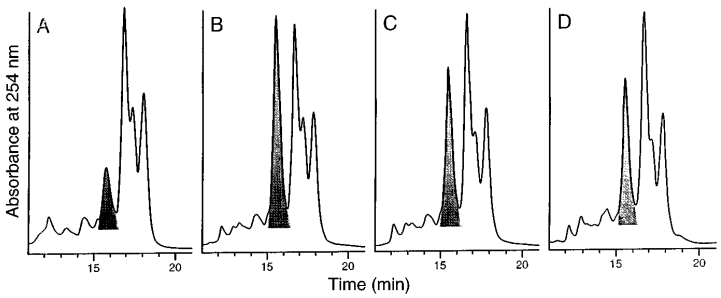

FIG. 2. Fractionation of highly unsaturated molecular species of rat hepatocyte PC as DAG–DNB derivatives. Reverse-phase HPLC on a Waters Nova Pak C18 column (Milford, MA) using methanol/isopropanol/glacial acetic acid (90:10:0.01, by vol). A: control; B: incubation for 20 min with 0.3 mM arachidonic acid; C and D: further incubation without arachidonic acid for 40 and 80 min, respectively. Shaded peak: 20:4-20:4 DAG–DNB. For abbreviations see Figure 1.

these newly synthesized molecular species. This is illustrated for 20:4-20:4 PC in Figure 2. On the basis of our previous data (15), we suggest that this reduction is primarily due to remodeling rather than degradation.

Remodeling of newly synthesized polyunsaturated molecular species. As expected from previous experiments (15), [U-^{14}C]glycerol was incorporated primarily into molecular species containing the exogenous fatty acids at the *sn*-1 and *sn*-2 positions. In Figure 3, the incorporation and redistribution of radioactivity in PC and PE molecular species are shown for control hepatocytes (ethanol only) and cells incubated in the presence of 18:2. Radioisotope data are compared to mass distribution data for the same molecular species. Uptake and redistribution in control cells followed a pattern similar to previously published data (14), except that in the present experiment highly unsaturated molecular species were as-

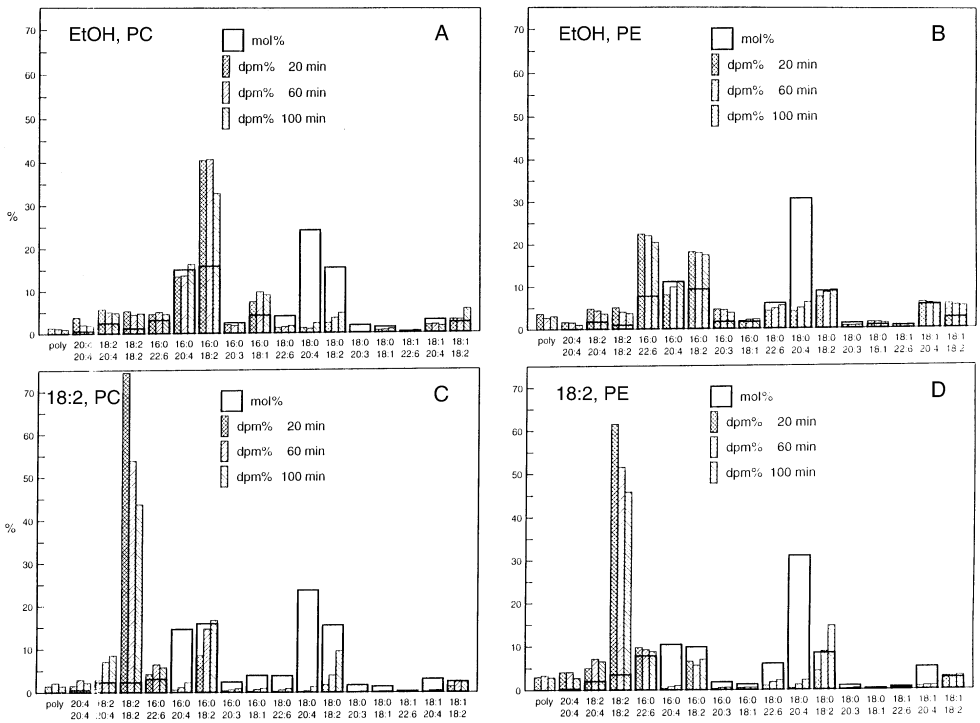

FIG. 3. Distribution of radioactivity from [U-^{14}C]glycerol in PC and phosphatidylethanolamine (PE) molecular species of rat hepatocytes incubated for 20 min with ethanol (A,B) or 0.3 mM linoleic acid (C,D), followed by further incubation for 40 and 80 min in the absence of labeled substrate, ethanol, and fatty acid. Polyunsaturated fractions emerging before the 20:4-20:4 peak were collected together (poly). Data are averages of two separate incubations and analyses. For abbreviations see Figure 1.

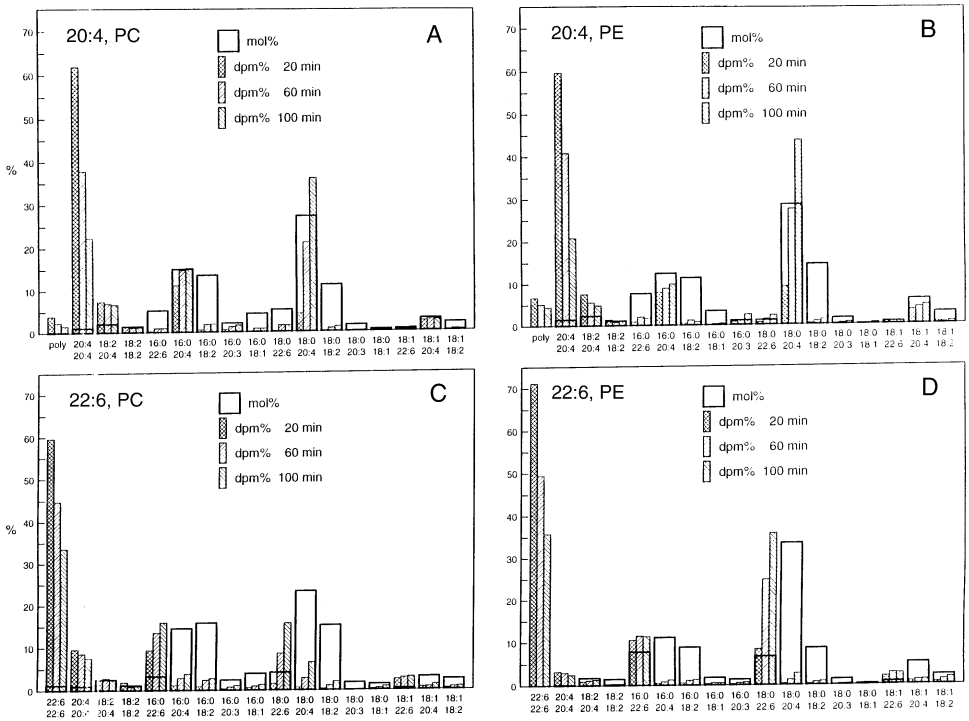

FIG. 4. Distribution of radioactivity from [U-^{14}C]glycerol in PC and PE molecular species of rat hepatocytes incubated for 20 min in the presence of 0.3 mM arachidonic (A,B) and docosahexaenoic acid (C,D), followed by further incubation for 40 and 80 min in the absence of labeled substrate and fatty acid. Polyunsaturated fractions emerging before the 20:4-20:4 peak in the 20:4 experiment (panels A,B) were collected together. The fraction identified as 20:4-20:4 in the 22:6 experiment (panels C,D) also contained 18:2-22:6 (Ref. 15). Data are averages of two separate incubations and analyses. For Abbreviations see Figure 1.

sayed separately and were found to exhibit very high specific activity. For both PC and PE, initial high specific radioactivity, decreasing after removal of the substrate, signifies *de novo* synthesis, whereas initially low but increasing radioactivity indicates generation by remodeling from precursor molecular species. In the case of 18:2, by far the highest percentage of glycerol label (74% in PC and 63% in PE) was originally present in the 18:2-18:2 molecular species. Remodeling of this molecular species was more rapid within PC where it apparently involved both the *sn*-1 acyl group leading to 16:0-18:2 and 18:0-18:2 PC and the *sn*-2 acyl group resulting in the formation of 18:2-20:4 PC. Remodeling of 18:2-18:2 PE occurred only at *sn*-1 and led to the formation of 18:0-18:2 PE as the major product.

Remodeling of both PC and PE molecular species was even more pronounced after incubation of hepatocytes in the presence of arachidonic and docosahexaenoic acids, resulting in the formation of 20:4-20:4 and 22:6-22:6 PC and PE (Fig. 4). Initially, 60–70% of the total radioactivity in PC and PE was in the 20:4-20:4 and 22:6-22:6 species, respectively, and remodeling occurred primarily by replacement of the *sn*-1 acyl groups with 18:0 and, to some extent, with 16:0. Reacylation with 18:0 was more pronounced in PE than in PC, resulting in an increase of radioactivity from less than 10% to about 45 and 37% of total radioactivity being associated with 18:0-20:4 PE and 18:0-22:6 PE, respectively (Fig. 4 B and D).

DISCUSSION

It is generally accepted that *de novo* biosynthesis of PC and PE *via* glycerophosphate (16) exhibits positional specificity which results in the formation of molecular species containing primarily saturated (plus monounsaturated) fatty acids at the *sn*-1 position and polyunsaturated (plus monounsaturated) fatty acids at the *sn*-2 position (reviewed in Refs. 1–4). However, Sundler *et al.* (25) first reported that, in the presence of excess fatty acids, hepatocytes synthesize molecular species containing the added fatty acid at both the *sn*-1 and *sn*-2 positions. Specifically, the addition of oleic and linoleic acids yielded relatively large proportions of dioleoyl and dilinoleoyl glycerophospholipids as well as trioleoyl- and trilinoleoylglycerols (25). Owing to the methodology available at that time, molecular species with a total of eight or more double bonds could not be identified, and subsequent studies were similarly limited (26–29).

Our present data suggest that *de novo* synthesis of glycerolipids by the Kennedy pathway is the main route of incorporation of excess fatty acids taken up from the media and that molecular species of both PC and PE containing the exogenous

fatty acid at both the *sn*-1 and *sn*-2 positions are the principal products. The highly unsaturated molecular species of both PC and PE are readily remodeled into more saturated species, primarily by replacement of the *sn*-1 acyl group with stearate (18:0). Because levels of radioactivity in PC and PE remained fairly constant after the [U-^{14}C]glycerol pulse, remodeling rather than degradation must have been the major metabolic conversion of the newly synthesized molecular species. This is in agreement with our earlier conclusions regarding PE turnover in rat hepatocytes (5,14) but in conflict with the results of pulse-chase experiments with labeled ethanolamine over a 24-h period. These data suggested that different molecular species of PE in rat hepatocytes are synthesized and degraded at different rates rather than being converted into one another through remodeling (30,31). Remodeling clearly occurs for the PE species synthesized in the present experiment.

It is important to consider that the concentrations of exogenous fatty acids used in the present study do not represent physiological levels, and hence our data constitute the results of model experiments. However, small amounts of very highly unsaturated molecular species, including 18:2-18:2, 20:4-20:4, 18:2-20:4 and 18:2-22:6 PC and PE, occur naturally in hepatocytes and other cells, and they exhibit a relatively high rate of turnover, most likely through *de novo* synthesis and remodeling (15,32). Recently we have shown that agonist-induced liberation of arachidonic acid from macrophage glycerolipids results in increased turnover not only through lysophospholipid reacylation but also through enhanced *de novo* synthesis of polyunsaturated PC molecular species, including 20:4-20:4 PC (32).

With respect to the large amounts of exogenous fatty acids taken up by hepatoctyes in the present study, we suggest that esterification of exogenous fatty acids, especially polyunsaturated fatty acids, into TAG may be the purpose of enhanced *de novo* glycerolipid synthesis. The synthesis of triarachidonoyl glycerol has been shown in HL-60 cells incubated with arachidonic acid (21), and we have observed a rapid and selective increase in arachidonic acid (from 1.7 to 15.4 mol%) in TAG of rat hepatocytes incubated with a mixture of palmitic, oleic, and arachidonic acids (5). Rat hepatocyte TAG exhibit rapid acyl turnover, even in the absence of exogenous fatty acids. Whether this occurs only through *de novo* synthesis and complete degradation or through deacylation–reacylation steps involving DAG or monoacylglycerols is not known at this time and deserves further study.

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Analysis of the Seed Oil of *Heisteria silvanii* (Olacaceae) —A Rich Source of a Novel C_{18} Acetylenic Fatty Acid

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ABSTRACT: Besides some usual fatty acids (FA), two conjugated ene-yne acetylenic FA [*trans*-10-heptadecen-8-ynoic acid (pyrulic acid) (7.4%), and *trans*-11-octadecen-9-ynoic acid (ximenynic acid) (3.5%)], a novel ene-yne-ene acetylenic FA [*cis*-7, *trans*-11-octadecadiene-9-ynoic acid (heisteric acid) (22.6%)], and 9,10-epoxystearic acid (0.6%) could be identified in the seed oil of *Heisteria silvanii* (Olacaceae). Two further conjugated acetylenic FA [9,11-octadecadiynoic acid (0.1%) and 13-octadecene-9,11-diynoic acid (0.4%)] were identified tentatively by their mass spectra. The FA mixture has been analyzed by gas chromatography/mass spectrometry (GC/MS) of their methyl ester and 4,4-dimethyloxazoline derivatives. The structure of heisteric acid was elucidated after isolation *via* preparative silver ion thin-layer chromatography and by various spectroscopic methods [ultraviolet; infrared; ^1H, ^{13}C nuclear magnetic resonance (NMR); ^1H-^1H and ^1H-^{13}C correlation spectroscopy]. To determine the position of the conjugated ene-yne-ene system, the NMR spectra were also measured after addition of the lanthanide shift reagent Resolve-Al EuFOD™. Furthermore, the triyglyceride mixture was analyzed by high-temperature GC and high-temperature GC coupled with negative chemical ionization MS. A glass capillary column coated with a methoxy-terminated 50%-diphenyl-50%-dimethylpolysiloxane was used for the separation of the triacylglycerol (TAG) species. No evidence of decomposition of the TAG species containing conjugated ene-yne-ene FA was observed. Twenty-six species of the separated TAG were identified by means of their abundant quasi molecular ion $[M - H]^-$and their corresponding carboxylate anions $[RCOO]^-$ of the fatty acids, respectively. The major molecular species of the TAG were found to be 16:0/18:1/18:1, 16:0/18:1/18:3 (heisteric acid), 17:2 (pyrulic acid)/18:1/18:1, 18:1/18:1/18:3 (heisteric acid). The TAG containing acetylenic FA showed an unexpected increase of the retention time in comparison to the TAG containing usual FA, thus making the prediction of the elution order of lipid samples containing acetylenic FA difficult.
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Abbreviations: ACN, acyl carbon number; BSTFA, *N,O*-bis(trimethylsilyl)-trifluoroacetamide; COSY, correlation spectroscopy; DEPT, distortionless enhanced polarization transfer; DMOX, 4,4-dimethyloxazoline derivatives; EUFOD, lanthanide shift reagent Resolve-Al EuFOD™ [tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionato)europium]; FA, fatty acid; FAME, fatty acid methyl ester; GC, gas chromatography; HTGC, high-temperature capillary gas chromatography; HTGC/NCI–MS, high-temperature capillary gas chromatography/negative chemical ionization–mass spectrometry; IR, infrared; $[M - H]^-$, quasi molecular ion; MS, mass spectrometry; NMR, nuclear magnetic resonance; SOP-50, methoxy-terminated 50%-diphenyl-50%-dimethylpolysiloxane; TAG, triacylglycerol; TLC, thin-layer chromatography; TMS, trimethylsilane; UV, ultraviolet.

The plant family Olacaceae can be divided into the three subfamilies Anacolosoideae, Olacoideae, and Schoepfioideae containing 30 genera with about 250 species. It occurs mainly in the tropical and subtropical hemisphere (1). Only a few Olacaceae species have been examined chemically, and a clear chemotaxonomical evaluation is already difficult. However, the samples analyzed up to now demonstrated that most of them yield oily seeds and that the lipid components were found to be unusual in that acetylenic fatty acids (FA), partially with additional keto and hydroxy groups (2–4), ricinoleic acid (2), very long-chain monounsaturated FA with up to 30 carbon atoms (2), and about 4% of all-*cis*-1,4-polyisoprene (5) could be identified. It is noteworthy that in some cases the seed triacylglycerols (TAG) were identical with those in the roots and the leaves of the Olacaceae plants (6).

In order to extend the knowledge of the seed oil chemistry of the Olacaceae, the species *Heisteria silvanii* (popular name: "casca-de-tatu") was included in our current research project on Brazilian seed oils. The native southern Brazil plant (7) is a medium-sized tree of 3–20 m height that yields single-seeded oily stone-fruits with a diameter of about 1.5 cm. The wood of the trees can be used for the production of high-quality flooring and firewood, but is not commercially explored (7).

Only a few literature references have been reported on the chemistry of the genus *Heisteria* (1,6), the largest genus of Olacaceae with 33 subspecies (8). In the seed oil of *H. elegans* (syn. *H. parvifolia*), 60% of oleic acid and 23% of an unknown FA were analyzed (1). The fresh fruits of *H. olivae* ("cacaito") contain the tropan alkaloid scopolamine and are used occasionally as a hallucinogenic drug in Venezuela (6).

The present paper deals with the analysis of the FA and TAG by spectroscopic methods, capillary gas chromatogra-

phy/mass spectrometry (GC/MS), high-temperature capillary gas chromatography (HTGC) and high-temperature capillary GC/negative chemical ionization mass spectrometry (HTGC/NCI-MS), respectively. Both high-temperature techniques were applied to a sample containing acetylenic FA for the first time. Furthermore, the isolation and structural elucidation of a novel C_{18} acetylenic FA from *H. silvanii* are described in detail. To the best of our knowledge, this is the first report on the chemistry of the plant *H. silvanii*.

MATERIALS AND METHODS

Seeds and oil extraction. The fruits from *H. silvanii* Schwacke were collected in December 1995 near the city of Porto Alegre, Rio Grande do Sul, Brazil. The air-dried, crushed seeds were extracted in a Soxhlet apparatus for 4 h with petroleum ether (40–60°C). After extraction, the solvent was removed by vacuum distillation at 30°C, flushed with nitrogen, and stored at −18°C until use. The yield was 46.6% of a pale-yellow oil.

Derivatization reactions. Transesterification to the fatty acid methyl esters (FAME) was carried out with 0.5 N sodium methoxide in anhydrous methanol as described by Christie (9). Silanization of the FAME mixture was made with *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) (Fluka, Buchs, Switzerland) (10). The 4,4-dimethyloxazoline derivatives (DMOX) were prepared by reaction of one part free FA mixture (obtained after hydrolysis of the oil with 1 N potassium hydroxide in 95% ethanol and subsequent acidification with HCl to pH 1) with five parts of 2-amino-2-methylpropanol for 1 h at 175°C (11).

Before the HTGC analysis, the total lipid mixture was derivatized with BSTFA to avoid contamination of the column with nonvolatile minor compounds. For this purpose, 1 mg sample was diluted in 1.75 mL pyridine, and 0.25 mL BSTFA was added. The mixture was allowed to react 20 min at ambient temperature, then the sample was diluted with pyridine and an aliquot was injected for HTGC analysis.

Alkaloid screening. For detection of alkaloids, the petroleum ether extract was analyzed using standard procedures that involve the use of the spray reagents Draggendorff, iodinoplatinate, and Marquis (12).

Thin-layer chromatography (TLC). For analytical purposes, commercial aluminum plates (5 × 10 cm) coated with 0.25 mm silica (Merck, Darmstadt, Germany) have been used. Preparative separations were carried out using glass plates (20 × 20 cm) coated with 0.6-mm silica layers and silica layers impregnated with silver nitrate (10%), respectively. To isolate the usual FAME fraction (FR-1) and the FAME fraction with acetylenic groups (FR-2), the total FAME was chromatographed using petroleum ether/diethyl ether (96:4, vol/vol) as solvent system. FR-2 was further subfractionated into the fractions AG-1 and AG-2 by using silver nitrate-impregnated silica plates and toluene as solvent system. The separation process was carried out in a refrigerator (4°C) within a tank containing a mixture of ice and NaCl. The bands of interest were scraped off [detected with ultraviolet (UV) light (254 nm) and 2′,7′-dichlorofluorescein] and eluted with diethyl ether. Monitoring of the individual fractions was carried out by GC, infrared (IR), UV, and nuclear magnetic resonance (NMR) spectroscopy.

GC and GC/MS analysis. A GC-17A (Shimadzu, Tokyo, Japan) equipped with a flame-ionization detector (260°C) and a split/splitless injector 260°C (split 1:100) with glass insert was used for separation and quantification of the FAME. Helium was carrier gas at an inlet pressure of 80 kPa. For peak integration, the software package Class CR-10 (Shimadzu) was used. Separation of the compounds was achieved on a BPX-70 (SGE, Weiterstedt, Germany) fused-silica capillary column (30 m, 0.22 mm i.d., 0.25 μm film thickness) using a temperature program (130–255°C, 2°C/min). GC/MS was done with a GCMS-QP5000 (Shimadzu) operating with an ionization energy of 70 eV and an interface temperature of 250°C. The separation conditions for the FAME and DMOX derivatives were the same as used in the GC analysis.

HTGC analysis of the seed oils. The HTGC system consists of a Carlo Erba Mega 5160 GC equipped with an on-column injector, a constant-flow pressure regulator (CP-CF 516) and a flame-ionization detector, which was fitted with a ceramic jet and heated to 400°C. Separation of the total lipid mixture was carried out on a Duran glass capillary column with a length of 10 m and 0.2 mm inner diameter. The capillary column was pretreated and coated with a 0.10 μm film of SOP-50 (50%-diphenyl-50%-dimethylpolysiloxane, methoxy-terminated), respectively. The column was conditioned at a temperature of 400°C (13). Further detailed descriptions of the preparation of high-temperature capillary columns were given in Ref. 14.

The oven temperature was programmed as follows: 1 min at 70°C, from 70 to 250°C at 12°/min, from 250 to 380°C at 4°/min. Hydrogen was employed as carrier gas with a linear velocity of 0.5 m/s.

Data acquisition was carried out on a Spectra Physics Labnet System (San Jose, CA) (Spectra Physics Integrator 4270 linked to a PC 3/86 data station).

HTGC/MS analysis of the seed oils. For HTGC/MS analyses a Carlo Erba Mega 5160 GC was coupled *via* a specially designed high-temperature interface (370°C) (15) to a Finnigan TSQ-700 mass spectrometer (San Jose, CA). The TSQ-700 was equipped with two corrosive gas stable turbo molecular pumps (Type 360C; Balzers-Pfeiffer GmbH, Asslar, Germany) at the source chamber (bottom and right flange) and one turbo molecular pump (Type 360, Balzers) at the analyzer chamber of the manifold of the mass spectrometer, respectively. This configuration provided sufficient vacuum capacity for the use of hydrogen as a carrier gas and chemical inertness against corrosive reagent gases. NCI was carried out using ammonia as an NCI reagent gas. The ion source was heated to a temperature of 180°C, filament current was 20 mA, electron multiplier voltage 1400 V, the conversion dynode was set to 15 kV, and the acquisition mass range was 200–1300 m.u. in 2 s. Separation during the HTGC/MS

analysis was achieved with the same type of column described above using the same temperature program.

Spectroscopic analyses of the oil and the isolated FAME fractions. The UV spectra were recorded from 400–200 nm in cyclohexane solution using a UV-2201 (Shimadzu) spectrophotometer. IR spectra were obtained with an FTIR-8101 (Shimadzu) in a liquid film. The ^{1}H NMR and ^{13}C NMR spectra were run in deuteriochloroform solution on a Varian VLX-200 (Palo Alto, CA) and on a Bruker (Karlsruhe, Germany) AC400 spectrometer with tetramethylsilane (TMS) as internal standard. The isolated heisteric acid methyl ester (ME) was examined by ^{1}H NMR, ^{1}H-^{1}H-2D shift correlation (COSY), ^{13}C NMR, distortionless enhanced polarization transfer (DEPT), and ^{1}H-^{13}C 2D heteronuclear shift correlation experiments on a Bruker AMX500 spectrometer. Additional spectra were measured after adding increasing amounts [up to 200 µL (20 mM in $CDCl_3$) in 500 µL of the total solution] of the lanthanide shift reagent Resolve-Al EuFOD™ 99+% (16,093-8; Aldrich Chemical Company, Milwaukee, WI).

RESULTS AND DISCUSSION

Preliminary analytical data on the seed oil. The UV spectrum exhibited absorption maxima at 282, 267, 255, 241 and 230 nm, indicating the presence of conjugated double/triple bond systems (16). In the IR spectrum, the absorption bands that appear in common seed oils such as soybean oil were accompanied by additional peaks at 2216 (vw), 2189 (vw), and 953 cm^{-1} (s). These are characteristic for triple bonds and double bonds in a *trans*-ene-yne linkage, respectively (16). The ^{13}C NMR (broadband decoupled and DEPT mode) signals in the range between δ 172–173.3 for the carbonyl atoms and δ 62–69 for the glycerol atoms indicated that the oil consisted mainly of TAG. No peaks for free FA, hydroxy FA, keto FA, or di- and monoacylglycerols could be detected under the experimental conditions. The occurrence of hydroxy and keto FA was already reported for other Olacaceae seed oils (2). In the range of the double-bond carbon signals (δ 100–150), a series of peaks at about δ 109 and 142–144 pointed to the presence of FA with conjugated double/triple bonds (4,17). This finding was confirmed by six quaternary (DEPT experiment) carbon signals (δ 79.3, 79.4, 84.8, 88.3, 88.5, and 92.6) that appeared in the range of the acetylenic groups between δ 78–93 (4,17). The signals at δ 32.9, 33.2, 19.27, and 19.3 could be attributed to methylene groups in allylic position to *trans* double bonds and to methylene groups in propargylic position to an acetylenic bond (18).

The presence of alkaloids could be proven neither by the spectroscopic data of the oil nor by the chemical test methods.

Identification of the individual FA of H. silvanii. In order to identify the FAME (Fig. 1; Table 1) and DMOX GC peaks (data not presented), various analysis and separation procedures were utilized. The peak numbers of the FA in the following text correspond with the numbers in Figure 1 and Table 1. The gas chromatograms of the FAME (Fig. 1) and

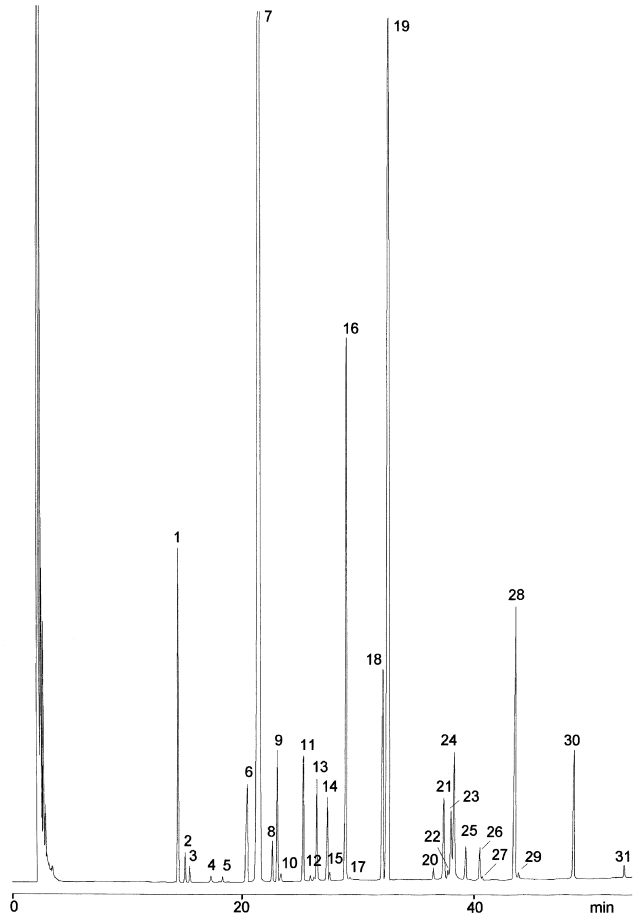


FIG. 1. Gas chromatogram of the fatty acid methyl esters of the seed oil of *Heisteria silvanii*. For experimental conditions, see the Materials and Methods section. The peak numbers correspond with those in Table 1.

DMOX derivatives, obtained with the BPX-70 column, did not show a noticeable difference in the total peak number and the individual peak areas (Table 1), respectively. Thus, it can be concluded that the DMOX derivatization procedure did not cause a significant degradation of the individual compounds under the reaction conditions.

Identification of the usual FA (peaks 1–7, 9, 11, 13, 14, 24, 28, 30, 31). FAME with known retention characteristics were identified by GC and GC/MS against authentic standards. In addition, the DMOX derivatives were examined by GC/MS, which enables the unequivocal location of the double bond(s) (11).

Identification of pyrulic and ximenynic acid (peaks 16, 18). TLC analysis of the total FAME revealed two main bands (FR-1 with $R_f = 0.88$ and FR-2 with $R_f = 0.77$). FR-2 was directly detectable under UV light, pointing to the presence of UV chromophores. For more detailed analysis, FR-1 and FR-2 were separated by preparative TLC. The GC analysis of FR-1 showed that this fraction contained the FA already identified as usual FA. The FR-2 consisted mainly of the FA 16, 18 and 19, respectively. By preparative argentation TLC, it was possible to separate the FR-2 into the fractions Ag-1 (R_f = 0.86) and Ag-2 ($R_f = 0.76$). GC analysis showed that the frac-

TABLE 1
The Fatty Acid Composition of the Seed Oil of *Heisteria silvanii*

Peak number	Fatty acid	Area%
1	16:0	2.93
2	16:1n-9	0.24
3	16:1n-7	0.14
4	17:0	<0.1
5	17:1n-8	<0.1
6	18:0	1.65
7	18:1n-9	46.62
8	Unknown 1	0.38
9	18:2n-6	1.19
10	Unknown 2	<0.1
11	18:3n-3	1.67
12	Unknown 3	<0.1
13	20:0	0.99
14	20:1n-9	0.91
15	Unknown 4	<0.1
16	17:2 (8a,10*t*)[a]	7.38
17	Unknown 5	<0.1
18	18:2 (9a,11*t*)	3.49
19	18:3 (*c*7,9a,11*t*)	22.63
20	18:2 (8a,11a)	0.10
21	Unknown 6	0.95
22	Unknown 7	<0.1
23	18:0 (9,10-epoxy)	0.64
24	24:0	1.27
25	18:3 (9a,11a,13)[a]	0.36
26	Unknown 8	0.39
27	Unknown 9	<0.1
28	26:0	3.83
29	Unknown 10	0.09
30	28:0	1.39
31	30:0	0.28

[a]Tentatively identified; a, triple bond; *c, cis* double bond; *t, trans* double bond.

tion Ag-2 contained the peaks 16 and 18, and that the fraction Ag-1 contained only the peak 19. The mass spectra of the FAME and DMOX derivatives of the peaks 16 and 18 were identical with those published for *trans*-10-heptadecen-8-ynoic acid (pyrulic acid) and *trans*-11-octadecen-9-ynoic acid (ximenynic acid) (4,17). The broad UV band at 229 cm^{-1} supported the conjugated ene-yne structure element (16). The presence of a *trans* double bond was also confirmed by the absorption band at 955 cm^{-1} in the IR spectrum (16). The ^1H (shift values and coupling constants) and ^{13}C NMR data of fraction Ag-2 were also in complete agreement with those of an analogous fraction from the seed oil of *Iodina rhombifolia* that contains pyrulic and ximenynic acid (17), previously analyzed in our laboratory. By considering all these spectroscopic data, the two FA from the fraction Ag-2 were unequivocally identified in terms of the chain length, and the position and stereochemical configuration of the ene-yne system. There was no evidence of an analogous derivative with *cis* configuration of the double bond as was detected in the seed oil of *Curupira tefeensis* (4) and *Santalum spicatum* (19).

Elucidation of heisteric acid (peak 19). The FAME isolated as fraction Ag-1 was subsequently elucidated as *cis*-7, *trans*-11-octadecadiene-9-ynoic acid ME by a combination of various spectroscopic methods. The UV spectrum of Ag-1 showed peak maxima at 280, 266, and 256 nm, suggesting the presence of a conjugated double/triple bond system with three unsaturated carbon-carbon linkages (16). The IR spectrum presented the following absorption bands: 3029, 2928, 2857, 2190, 1742, 1717, 1456, 1437, 1260, 1199, 1169, 953, and 727 cm^{-1}. The absorption band at 953 cm^{-1} (m) was assigned to a *trans* double bond in conjugation with a triple-bond system (16). Only a very weak band for an acetylenic bond was observed at 2190 cm^{-1}. The mass spectrum of the FAME of peak 19 (Fig. 2) revealed a series of highly unsaturated hydrocarbon fragments [*m/z* 41, 55, 67, 79, 91 (base peak), 105, 117, etc.] in the low-mass range. These ions are known as the result of rearrangement reactions of the unsaturated system in the ion source and are typical for highly unsaturated FAME (20) and FAME with acetylenic bonds (21). The molecular ion at *m/z* 290 suggested a FAME derivative with a C_{18} chain and four double-bond equivalents due to a molecular formula of $C_{19}H_{30}O_3$, respectively. Other weak ions in the higher mass range were found at *m/z* 259 ($M^+ - OCH_3$), 247 ($M^+ - C_3H_7$), 233 ($M^+ - C_4H_9$), 219 ($M^+ - C_5H_{11}$), and 205 ($M^+ - C_6H_{13}$). The latter ion series (*m/z* 247, 233, 219, 205) was considered to arise from methylene cleavages at the CH_3 end of the FA chain. The fact that this series was not further continued and that the ion at *m/z* 219 (allylic cleavage) was the most intense in this mass range suggested that the conjugated unsaturated system could be located at the n-7 end of the hydrocarbon chain (Fig. 2). Similar mass spectrometric characteristics were reported for a naturally occurring conjugated tetraenoic acid (22). It is noteworthy that the mass spectrum of Ag-1 lacked the prominent even-numbered fragment ions which are typical for acetylenic and conjugated olefinic-acetylenic FAME (23). This can be explained by the fact that the triple bond of Ag-1 is located between two double bonds, and thus the fragmentation mechanism observed for FAME that have an acetylenic system in direct neighborhood of a methylene group must be suppressed.

As the mass spectra of the DMOX derivatives of acetylenic FA in some cases are easily interpretable in terms of the position of the unsaturated system (4,17,24,25), the spectrum of the corresponding DMOX derivative of peak 19 was examined carefully (Fig. 2). The molecular ion was detected at *m/z* 329, which is in agreement with its FAME derivative. In the low-mass range, the typical intense ion peaks for the DMOX derivative at *m/z* 113 (McLafferty rearrangement) and 126 (cyclization-elimination reaction) were detected (11). The expected ion series of *m/z* 126 + 14n m.u. (140, 154, 168, ...), representing the cleavage at each methylene group, was not found to be well defined. The theoretically expected ions at *m/z* 140 and 154 were accompanied by other intense ions in the same region. Instead of the expected ion peak at *m/z* 168, peaks at *m/z* 164 and 166 could be observed. Comparison with the literature data of ene-yne derivatives (25) suggested that for the ene-yne-ene structure at C-7 of Ag-1 an ion series *m/z* 168-180-194-204-218-230-244-258 (see Fig. 2) could theoretically be expected. However, the

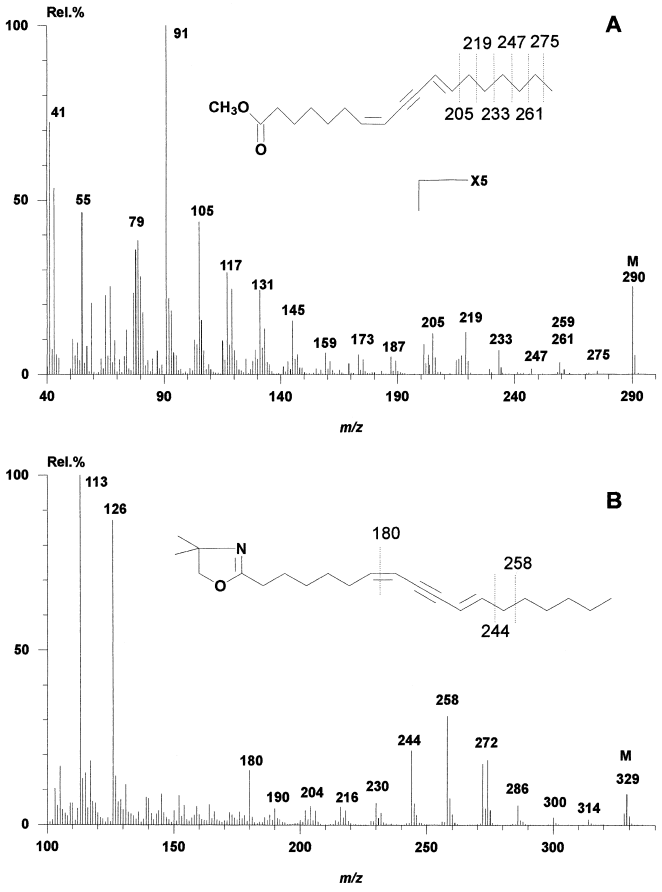


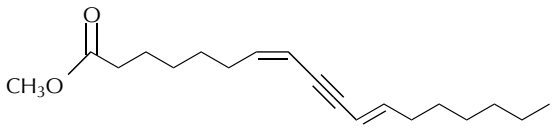
FIG. 2. The electron impact mass spectra of the methyl ester (A) and the 4,4-dimethyloxazoline derivative (B) of heisteric acid (*cis*-7, *trans*-11-octadecadiene-9-ynoic acid).

measured spectrum showed that this peak sequence appeared only partially. The ion at *m/z* 180 was the most intense in the region between *m/z* 140 and 230. This peak theoretically represents the ion produced by a cleavage between C-6 and C-7 of the FA chain. Instead of the ion at *m/z* 194, an unexpected peak at *m/z* 190 was observed. The following ion series at *m/z* 204-218-230-244-258 appeared as predicted, and the latter signal was found to be the third-most intense in the spectrum. This can be explained by the energetically preferred allylic cleavage (Fig. 2). The following ions up to the molecular ion showed the regular 14 m.u. ion series. These mass spectral data demonstrated that the DMOX derivative of Ag-1 can be helpful in locating the unsaturated system but that the complete identification of the individual unsaturated carbons is not possible. Obviously, the appearance potential of the conjugated system of Ag-1 must be similar to that of the oxazoline ring. Thus, the oxazoline ring and the unsaturated ene-yne-ene system can compete as preferred ionization center and the suppression of the hydrogen migration by the oxazoline ring during the ionization does not work as well as in the case of other unsaturated FA (26). Thus, the fragmentation reactions do not follow completely the rules established for ene-yne FA (4,17,24,25).

For further structure analysis, the NMR spectra were examined in detail. The ^{1}H and ^{13}C NMR spectral data are summarized in Tables 2 and 3, respectively. The assignment of the individual signals was possible by applying the chemical shift theory, by interpretation of the coupling patterns, by ^{1}H-^{1}H and ^{1}H-^{13}C chemical shift correlation experiments (COSY and HETCOR), by DEPT experiments, and by using the lanthanide shift reagent Resolve-Al EuFOD™ 99+%. Consistent with the proposed molecular formula, the NMR data revealed the presence of 10 aliphatic methylenes (one of them in allylic position to a *trans* and the other one to a *cis* double bond), four olefinic methines, two acetylenic carbons, two methyl groups, and one carbonyl group.

The ^{1}H NMR spectrum revealed nine signal groups (Table 2). It is noteworthy that the signal for the methylene group in allylic position to the *cis* double bond (C-6) appeared at the same shift value (δ 2.3) as the signal for the methylene group at C-2. The methylene group in allylic position to the *trans* double bond (C-13) arose at δ 2.1. In FA with isolated double bonds, the corresponding signal is found at higher field (δ 2.0). Obviously, the conjugated system caused a downfield shift on the methylene hydrogens in the allylic position. This was already observed in the ^{1}H NMR spectrum of the seed oil of *Sebastiana brasiliensis* that contains the conjugated α-parinaric acid (27). The COSY experiment demonstrated coupling from the allylic methylene signal at δ 2.3 through the olefinic proton signals at δ 5.85 [1H, *J* = 10.8(*d*); 7.4(*t*) Hz] to δ 5.55 [1H, *J* = 10.8(*d*) Hz].

TABLE 2
The ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) Data of Heisteric Acid (signals for the methylate group omitted)



Carbon number	^{13}C NMR signal	^{1}H NMR signal
1	174.32	—
2	34.10	2.3 (4H, *m*)[a]
3	24.85	1.6 [2H, *J* = 7.5(*tt*) Hz]
4	28.81[b]	1.3–1.4 (12 H, *m*)
5	28.88[b]	1.3–1.4 (12 H, *m*)
6	30.07	2.3 (4H, *m*)[a]
7	142.96	5.85 [1H, *J* = 10.8(*d*); 7.4(*t*) Hz]
8	109.5	5.55 [1H, *J* = 10.8(*d*) Hz]
9	92.66	—
10	84.9	—
11	109.76	5.65 (1H, d, *J* = 15.8 Hz)
12	144.58	6.15 [*J* = 15.8(*d*); 7.2(*t*) Hz]
13	33.26	2.1 [2H, *J* = 7.5(*q*) Hz]
14	28.72[b]	1.3–1.4 (12 H, *m*)[a]
15	28.56[b]	1.3–1.4 (12 H, *m*)[a]
16	31.74	1.3–1.4 (12 H, *m*)[a]
17	22.67	1.3–1.4 (12 H, *m*)[a]
18	14.16	0.9 [3H, *J* = 7.1(*t*) Hz]

[a]Signals not resolved.
[b]Signals may be reversed; d, double bond; a, triple bond; *m*, multiplet; *tt*, triplet of triplets; *t*, triplet; *d*, doublet; *q*, quartet.

TABLE 3
Mass Spectral Data and Identification of the Triacylglycerol Composition of the *Heisteria silvanii* Seed Oil by HTGC/NCI–MS

Peak number	[M – H] (*m/z*)	$RCOO^{-}$,[a] (*m/z*)	Molecular weight	Triacylglycerol[a]	ACN:*n*[b]
1	857	255 / 281 / 281	858	16:0 / 18:1 / 18:1	52:2
2	855	253 / 281 / 281	856	16:1 / 18:1 / 18:1	52:3
3	869	267 / 281 / 281	870	17:1? / 18:1 / 18:1	53:3
4	839	255 / **261** / 281	840	16:0 / **17:3** / 18:1	**51:4**
5	889	255 / 281 / 281	886	16:0 / 18:1 / 18:1	52:2
6	851	255 / 281 / **275**	852	16:0 / 18:1 / **18:3**	**52:3**
	883	281 / 281 / 281	884	18:1 / 18:1 / 18:1	54:3
7	881	281 / 281 / 279	882	18:1 / 18:1 / 18:2	54:4
8	879	281 / 279 / 279	880	18:1 / 18:2 / 18:2	54:5
9	865?	—	866?	n.i. / n.i. / n.i.	—
10	865	**263**/ 279 / 279	866	**17:2** / 18:1 / 18:1	**53:4**
11	879	283 / 281 / **275**	880	18:0 / 18:1 / **18:3**	**54:4**
12	877	281 / 281 / **275**	878	18:1 / 18:1 / **18:3**	**54:5**
13	879	281 / 281 / 277	880	18:1 / 18:1 / **18:2**	**52:4**
14	847	**263** / **263** / 281	848	**17:2** / **17:2** / 18:1	**52:5**
15	859	**263** / 281 / **275**	860	**17:2** / 18:1 / **18:3**	**53:6**
16	861	**263?** / **277** / 281	862	**17:2?** / **18:2** / 18:1	**53:5**
	907	281 / **275** / 311	908	18:1 / **18:3** / 20:0	**56:4**
17	905	281 / **275** / 309	906	18:1 / **18:3** / 20:1	**56:5**
18	871	281 / **275** / **275**	872	18:1 / **18:3** / **18:3**	**54:7**
19	873	281 / **277** / **275**	874	18:1 / **18:2** / **18:3**	**54:6**
20	969	281 / 279 / 367	970	18:1 / 18:1 / 24:0	60:2
21	1113[c]	—	1114[c]	—	—
22	997	281 / 281 / 395	998	18:1 / 18:1 / 26:0	62:2
23	963	281 / **275** / 367	964	18:1 / **18:3** / 24:0	**60:4**
24	979	**263** / 281 / 395	980	**17:2** / 18:1 / 26:0	**61:3**
25	1025	281 / 281 / 423	1026	18:1 / 18:1 / 28:0	64:2
26	991	281 / **275** / 395	992	18:1 / **18:3** / 26:0	**62:4**

[a]The regiospecific distribution of the fatty acyl residues has not been differentiated. Fatty acids with acetylene bonds are bolded.
[b]ACN is the total acyl carbon number and *n* the combined number of double bonds and triple bonds. Triacylglycerols with acetylene acyl groups are bolded.
[c]Compound has no triglyceride structure. HTGC/NCI–MS, high-temperature capillary gas chromatography/negative chemical ionization–mass spectrometry.

The value of about 11 Hz for the coupling constants within the latter double bond signals demonstrated, that their stereochemical configuration must be *cis*. The observed shift values, the coupling patterns, and the fact that no other couplings outgoing from δ 5.55 could be detected in the COSY spectrum demonstrated that this *cis*-olefinic structure element must be linked with the acetylenic group. The following partial structure was thus confirmed: $–CH_2–CH=CH$(*cis*)–C≡C–. The COSY experiment demonstrated moreover a coupling from the second allylic methylene signal at δ 2.1 through the olefinic proton signals at δ 6.15 [1H, J = 15.8(*d*); 7.2(*t*) Hz] to δ 5.65 [1H, J = 15.8(*d*) Hz]. The high value of about 16 Hz for the coupling constants within the double-bond system signals revealed here that its stereochemical configuration must be *trans*. Analogous to the *cis* double-bond protons, the observed shift values, the coupling patterns, and the lack of other couplings outgoing from δ 5.65 showed that the *trans*-olefinic structure element must also be linked directly with the acetylenic group. As the allylic methylene signals at δ 2.1 and 2.3 showed further couplings with other methylene groups, the partial structure $–CH_2–CH_2–CH=CH$(*cis*)–C≡C–CH=CH(*trans*)–$CH_2–CH_2–$ was proven.

Since the methylene signals in the ^{1}H NMR and COSY spectra were not completely resolved, it was not possible to identify the relative position of the conjugated ene-yne-ene system in the FA chain. Consequently, the NMR experiments were completed by using the lanthanide shift reagent Resolve-Al EuFOD™ [tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionato)europium] in increasing concentrations. The nucleophilic part of the FAME can build a reversible complex with the lanthanide central atom and the chemical shifts of the protons in neighborhood to this complex increase with the amount of EuFOD™ (28). The ^{1}H NMR spectra obtained by addition of 100 μL of the shift reagent (20 mM in $CDCl_3$) to 500 μL of the $CDCl_3$ solution of Ag-1 are shown in Figure 3. In comparison to the original spectrum (Fig. 3), four new signals could be observed. The COSY spectrum obtained with the same solution revealed clearly the linear array of the hydrogen atoms from C-2 to C-8 (Fig. 4). Coupling was observed in the following sequence: δ 4.0 (H-2)-2.7 (H-3)-1.85 (H-4)-1.70 (H-5)-2.45 (H-6)-5.85

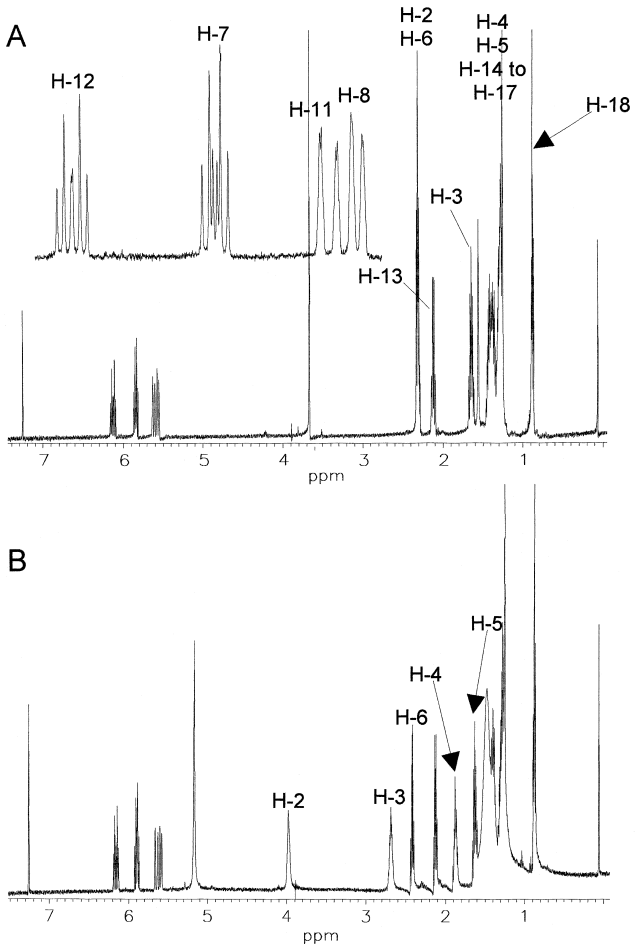


FIG. 3. The ^{1}H nuclear magnetic resonance spectra of heisteric acid (*cis*-7, *trans*-11-octadecadiene-9-ynoic acid) methyl ester recorded at 500 MHz in $CDCl_3$ solution (A) and after addition of 100 µL of Resolve-Al EuFOD™ 99+% (Aldrich Chemical Company, Milwaukee, WI) (B).

(H-7)-5.55 (H-8). These data demonstrated unambiguously that the conjugated unsaturated system begins at C-7 with the *cis* double bond. Considering all the other spectroscopic data, the structure of Ag-1 was thus elucidated as *cis*-7, *trans*-11-octadecadiene-9-ynoic acid ME. To our knowledge, this compound was, up to now, neither isolated from natural sources nor reported as a synthetic derivative. Since it occurs as one of the main compounds in the seed oil of *H. silvanii,* we propose the trivial name *heisteric acid*.

Further structure confirmation was obtained by ^{13}C NMR and HETCOR experiments (Table 2). The ^{13}C signals for the methylene carbon in allylic position to the *cis* and *trans* double bond appeared at δ 30 and 33.3, respectively. In usual *cis* unsaturated FA such as oleic acid, the corresponding ^{13}C signals appear at about δ 27 (18). The shift value for the second signal was again comparable to the corresponding signal in *trans* FA with the double bond at a similar chain position (18). Obviously, the signal for the methylene carbon in allylic position to the *cis* double bond suffered a significant downfield shift that was also observed in the ^{1}H spectrum. The signal pattern for the double (δ 109.5, 109.8, 143, 144.6)- and triple (δ 92.7, 84.9)-bond carbons demonstrated the unsymmetrical nature of the conjugated system. The lack of a ^{13}C signal for propargylic methylene groups at about δ 19 (18) further confirmed that the acetylenic bond must be located between the two double bonds.

Identification of 9,10-epoxystearic acid [peak 23 (Fig. 1)]. The mass spectrum of peak 23 (see Table 1) was found to be identical with that of 9,10-epoxystearic acid ME. Saturated epoxy esters produce mass spectra, the interpretation of which is so straightforward that epoxidation and MS form an established procedure to locate double bonds (29). After reaction with the silylation reagent mixture, peak 23 disappeared and a new peak eluted at lower temperature. Its mass spectrum was identical with that of 9,10-diTMS octadecanoic acid ME. Thus, the identity of peak 19 with 9,10-epoxystearic acid ME was unambiguously confirmed.

Information on other minor peaks (peaks 8, 10, 12, 15, 17, 20–22, 25–27, 29). Peaks 20 and 25 were identified tentatively as 9,11-octadecadiynoic acid and 13-octadecene-9,11-diynoic acid by comparison of their mass spectra of the FAME and DMOX derivatives with the spectra of the same derivatives published previously (4). A series of other unknown minor peaks was detected during the GC and GC/MS analyses. Their FAME mass spectra showed highly unsaturated hydrocarbon fragments in the low-mass range and no molecular ion was detectable. Unfortunately, the DMOX spectra did not provide further valuable structure information. From the GC and MS data, it is probable that these compounds are highly unsaturated and consist in C_{17} and C_{18} chains.

High-temperature GC and high-temperature GC/MS analyses. To obtain information about the FA distribution of the intact lipid species in the seed oil of *H. silvanii,* the sample was investigated by HTGC and HTGC/NCI-MS. In HTGC the use of polarizable stationary phases with a high content of phenyl groups is well known. The distinct selectivity of this type of stationary phase to double bonds facilitates a detailed investigation of neutral lipids. Separation of TAG according to their acyl carbon number (ACN) and within each ACN according to the number of double bonds could be achieved, whereby the retention time increased with the increasing number of double bonds (30–32). Therefore, HTGC analysis of the seed oil of *H. silvanii* was carried out on a glass capillary column coated with a thin film of a methoxy-terminated SOP-50. This stationary phase was designed for the special purposes of HTGC (13,33) and yielded, in the long-term, high-temperature stable glass capillary columns with high inertness and low bleeding even at high temperatures (13). The resulting FID chromatogram (Fig. 5) showed one main peak (peak 6) and one peak with medium height (peak 12); both towered above a number of minor compounds. No evidence of decomposition of the TAG species containing conjugated ene-yne-ene FA, in comparison to seed oils which were rich in TAG containing conjugated ene-ene-ene FA (Aichholz, R., Spitzer, V., and Lorbeer, E., 1997, unpublished), was observed.

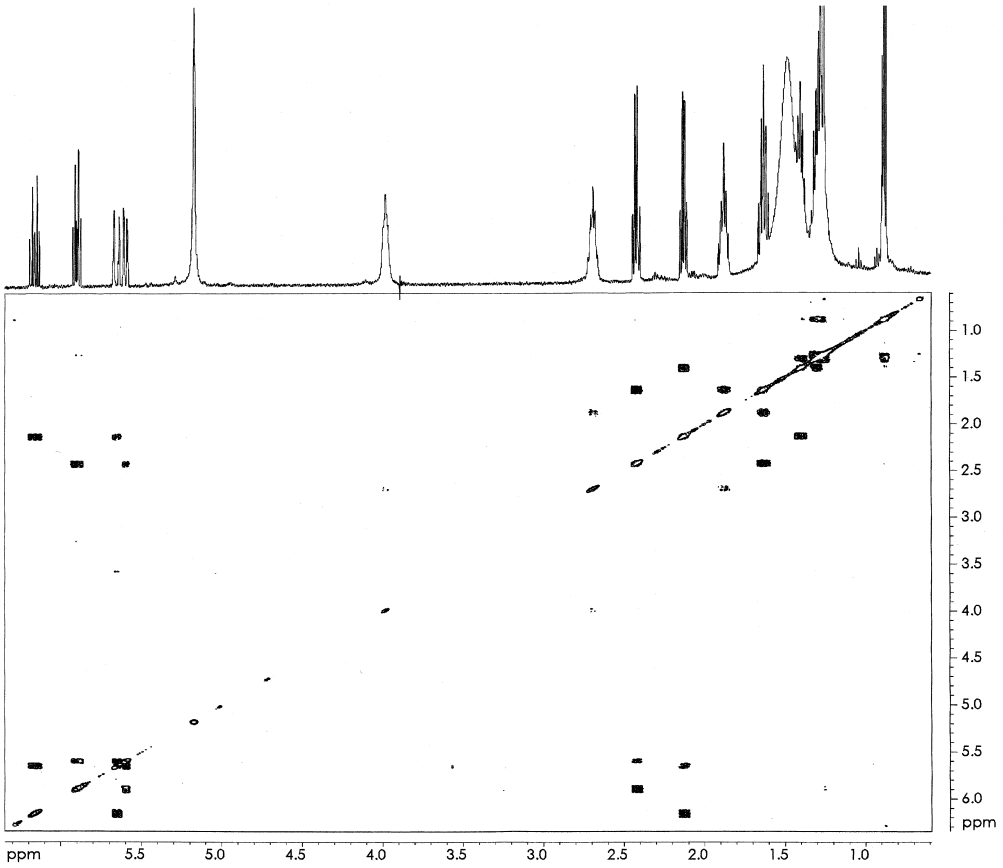


FIG. 4. The ^{1}H-^{1}H two-dimensional shift correlation nuclear magnetic resonance spectrum of heisteric acid (*cis*-7, *trans*-11-octadecadiene-9-ynoic acid) methyl ester recorded at 500 MHz after addition of 100 µL of Resolve-Al EuFOD™ 99+%. See Figure 3 for company source.

HTGC/NCI-MS was carried out, and the composition of the acyl moieties of 26 lipid species of *H. silvanii* was determined (Table 3). The peak pattern of Figure 5 showed an unusual retention behavior on the SOP-50 column of the investigated TAG. For instance, peak 6 consists of two TAG species with the ACN 52 and 54 containing the FA acyl groups 16:0/18:1/18:2(acetylene) and 18:1/18:1/18:1, respectively. Obviously, the presence of an acetylenic FA in the lipids species results in an unexpected increase of the retention time. A further special feature in the elution pattern is the occurrence of peaks 13 and 19. Both compounds eluted after a compound with a higher unsaturation (see Table 3), respectively. Thus, a prediction of the elution order of lipid samples containing acetylenic FA is difficult and needs identification with authentic standards and/or additional information about the constitution of the separated lipid species *via* HTGC/MS analysis.

HTGC/NCI-MS using ammonia as a reagent gas yielded characteristic mass spectra for the TAG with abundant ions at $[M - H]^-$ and $[RCOO]^-$ (34,35). Base peak for all TAG was the quasi molecular ion $[M - H]^-$. The mass spectra of peaks 12 and 26 (see Table 3) are shown in Figures 6 and 7, respectively. The occurrence of prominent quasi molecular ions enables the easy localization of the individual lipid species up to a molecular ion at *m/z* 1026 (see Fig. 8). However, the interpretation of the mass spectra of minor compounds was sometimes disturbed by uncharacteristic ions in the mass range between *m/z* 257 and 269 (see Fig. 6). The presence of a carboxylate anion at *m/z* 281, corresponding to the FA 18:1 $[RCOO]^-$, appeared in all identified lipid species and is in good agreement with the analysis of the total FA composition *via* their ME as listed in Table 1. The identification of the lipid species containing the esterified unusual long-chain FA chain 24:0 ($[RCOO]^-$ = *m/z* 367), 26:0 ($[RCOO]^-$ = *m/z* 395), 28:0 ($[RCOO]^-$ = *m/z* 423) and those containing the esterified acetylene FA 17:2 ($[RCOO]^-$ = *m/z* 263), 18:2 ($[RCOO]^-$ = *m/z* 277) and 18:3 ($[RCOO]^-$ = *m/z* 275) was also possible. The method failed only in the identification of FA with a content <1% in the total FA composition. Furthermore, the regiospecific distribution of the FA (*sn*-2 vs. *sn*-1/3), differentiation of the position of the unsaturation along the FA chain and differentiation of *cis/trans*-isomers cannot be distinguished with this method.

Chemotaxonomic and nutritional considerations. All of the main C_{17} and C_{18} acetylenic FA from the seed oil of *H. silvanii* have their conjugated unsaturated system at the n-7

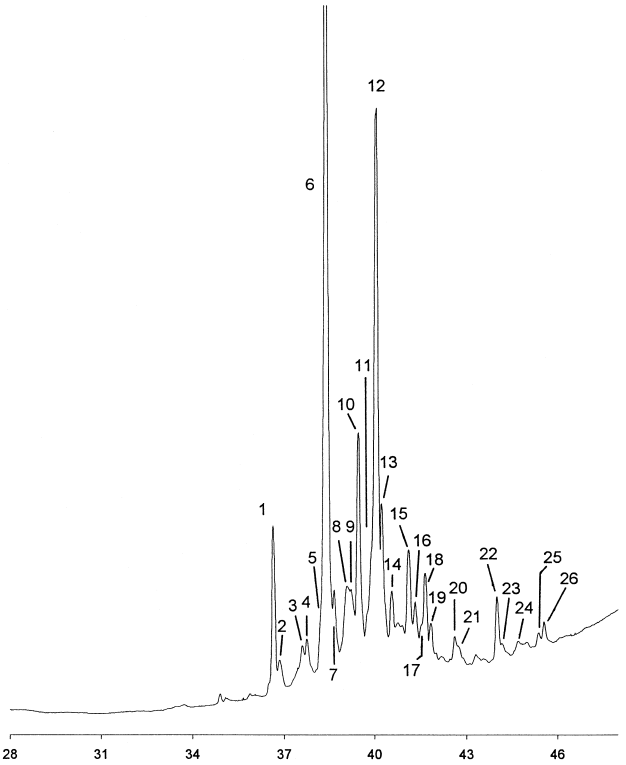


FIG. 5. Flame-ionization detection chromatogram of the seed oil of *Heisteria silvanii* achieved by high-temperature gas chromatography. Analytical details are described in the Materials and Methods section. Peak numbers refer to Table 3.

terminal of the hydrocarbon chain. Up to now, this type of compounds was only found in the two closely related plant families Santalaceae and Olacaceae (both members of Santalales), and they are therefore of chemotaxonomic importance (1). It was observed that the Olacaceae show a preponderance of FA with two acetylenic groups (6) which is not the case in the seed oil of *H. silvanii*. The 9,10-epoxystearic acid, which could be an intermediate product during the biosynthesis of higher unsaturated FA outgoing from oleic acid (36), has been detected already as a minor compound in some other seed oils (2) without a close relationship. There was no evidence of 9-hydroxystearic acid that has been found in the oils of *Ongokea gore* (37) and *I. rhombifolia* (17) that both belong to the Santalales. The presence of relative high amounts of very long-chain saturated FA without being accompanied by the homologous monounsaturated FA was not reported before for an Olacaceae seed oil.

From the nutritional point of view, the seeds and the oil of *H. silvanii* cannot be recommended as the physiological properties of the novel heisteric acid are still unexplored. Furthermore, in a recent work it was demonstrated that conjugated acetylenic FA as xymenynic acid can have a significant influence on the prostaglandin metabolism (38).

ACKNOWLEDGMENTS

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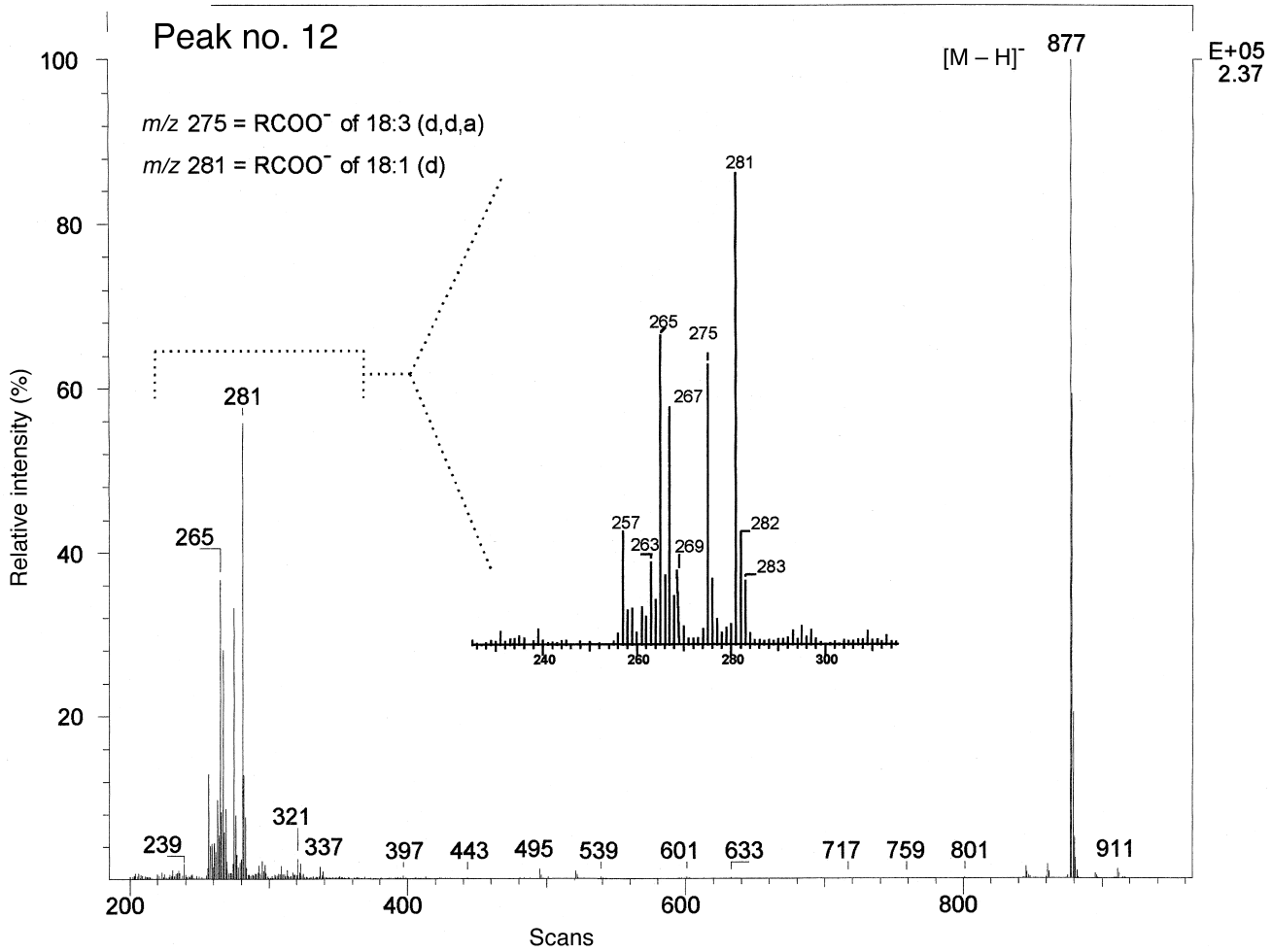

FIG. 6. Negative chemical ionization–mass spectrum obtained from peak 12. Analytical details are described in the Materials and Methods section. The peak number refers to Table 3; d = double bond; a = triple bond.

Spectra of Methyl Oleate, Methyl Linoleate and Methyl Linolenate, *Acta Chem. Scand. 13,* 845–847.
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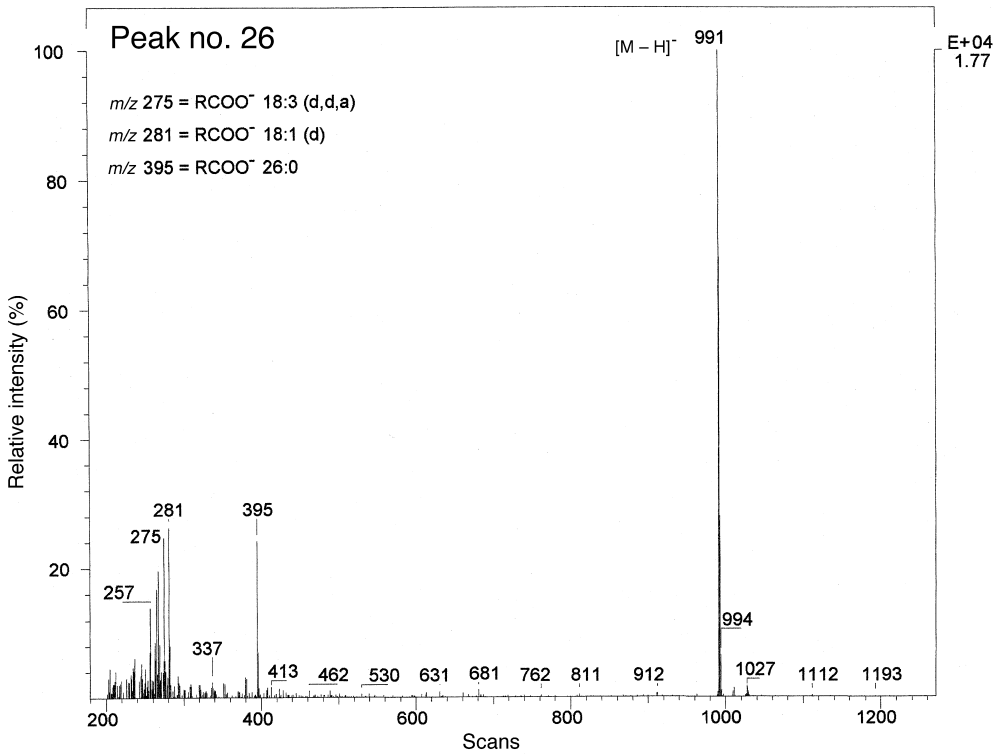

FIG. 7. Negative chemical ionization–mass spectrum obtained from peak 26. Analytical details are described in the Materials and Methods section. Peak number refers to Table 3; d = double bond; a = triple bond.

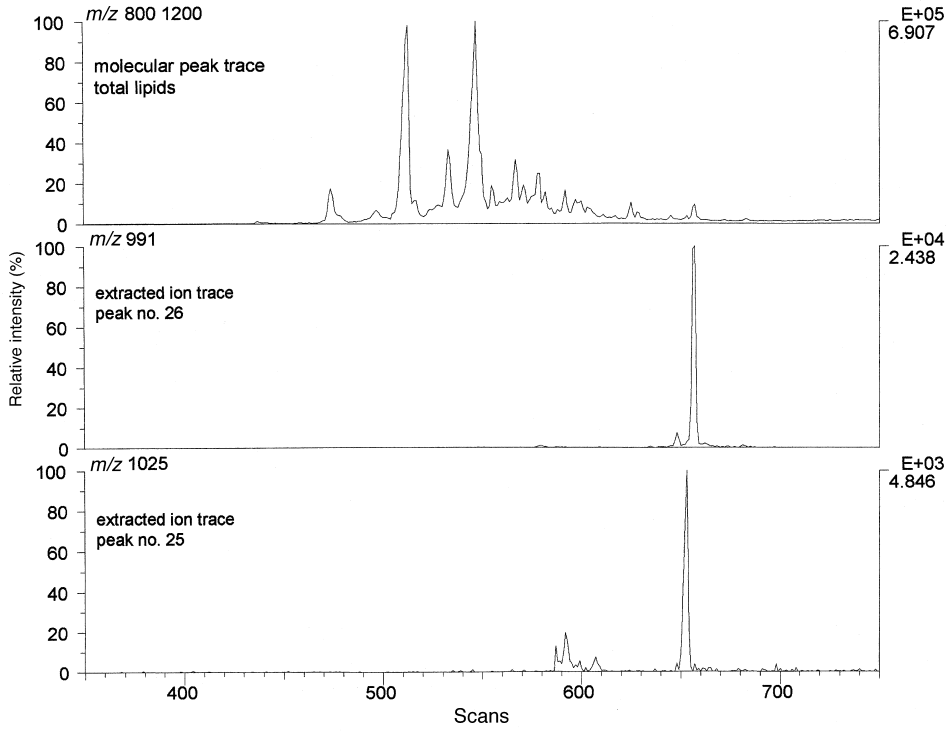

FIG. 8. Molecular ion chromatogram (*m/z* 800–1200) of the seed oil of *Heisteria silvanii* and the single ion chromatograms *m/z* 991 and *m/z* 1025. Separation of the triacylglycerols was performed by high-temperature gas chromatography whereby the components were detected by negative chemical ionization mass spectrometry. The molecular ion chromatogram is the ion current of the selected mass range *m/z* 800–1200. This trace has the advantage of a better signal-to-noise ratio in comparison with the total ion current. The single-ion chromatograms of *m/z* 991 and *m/z* 1025 corresponded to the $[M - H]^-$ ions of the peaks 25 and 26, respectively. By using single-ion chromatograms, localization of the respective triacylglycerol species was facilitated. Analytical details are described in the Materials and Methods section. The peak numbers refer to Table 3.

Tierreich (eine Übersicht) II: Trans-ungesättigte, Alkin-, Hydroxy-, Epoxy-, Oxo-, Cyclopropan- und Cyclopropen-Fettsäuren, *Fette Seifen Anstrichm. 74,* 541–550.
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Lipid Specificity and Location of the Sterol Carrier Protein-2 Fatty Acid-Binding Site: A Fluorescence Displacement and Energy Transfer Study[1]

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ABSTRACT: Although it was recently recognized that sterol carrier protein-2 (SCP-2) interacts with fatty acids, little is known regarding the specificity of SCP-2 for long-chain fatty acids or branched-chain fatty-acid-like molecules. Likewise the location of the fatty-acid binding site within SCP-2 is unresolved. A fluorescent *cis*-parinaric acid displacement assay was used to show that SCP-2 optimally interacted with 14–22 carbon chain lipidic molecules: polyunsaturated fatty acids > monounsaturated, saturated > branched-chain isoprenoids > branched-chain phytol-derived fatty acids. In contrast, the other major fatty-acid binding protein in liver, fatty-acid binding protein (L-FABP), displayed a much narrower carbon chain preference in general: polyunsaturated fatty acids > branched-chain phytol-derived fatty acids > 14- and 16-carbon saturated > branched-chain isoprenoids. However, both SCP-2 and L-FABP displayed a very similar unsaturated fatty-acid specificity profile. The presence and location of the SCP-2 lipid binding site were investigated by fluorescence energy transfer. The distance between the SCP-2 Trp^{50} and bound *cis*-parinaric acid was determined to be 40 Å. Thus, the SCP-2 fatty-acid binding site appeared to be located on the opposite side of the SCP-2 Trp^{50}. These findings not only contribute to our understanding of the SCP-2 ligand binding site but also provide evidence suggesting a potential role for SCP-2 and/or L-FABP in metabolism of branched-chain fatty acids and isoprenoids.
Lipids 32, 1201–1209 (1997).

Eukaryotic cells contain a variety of lipid transfer proteins (1–3). While some of these, such as fatty-acid binding proteins (FABP) and fatty acyl CoA binding protein, appear localized primarily to the cell cytoplasm (2,3), others such as sterol carrier protein-2 not only are found in cytoplasm but also are highly enriched in peroxisomes (4–7). The origin and function of the cytoplasmic form of SCP-2 are unclear at this time. Furthermore, the ligand specificities of many of the lipid transfer proteins are quite broad. This range of specificity is best exemplified by SCP-2, also called the nonspecific lipid transfer protein. SCP-2 is a soluble 13.2 kDa basic protein found in all mammalian tissues examined (8–13). Studies *in vitro* indicate that SCP-2 transfers nearly all phospholipids tested (except cardiolipin) (14,15), glycosphingolipids and gangliosides (16), and sterols (1,8,17–26). Furthermore, SCP-2 stimulates a variety of terminal enzymatic reactions in cholesterol biosynthesis and cholesterol esterification *in vitro* (8,9,18). Although the physiological function(s) of SCP-2 may relate to its ability to transfer the above lipids, this has been established only for cholesterol in permanently transfected L-cells (27) and in transiently transfected Chinese hamster ovary cells (28).

The location of the SCP-2 ligand binding site has not been identified. While the structure of apo SCP-2 has been determined by nuclear magnetic resonance (NMR) spectroscopy, that of the holo-SCP-2 has not been reported (29). Resolution of an SCP-2 ligand binding site has been difficult owing to: (i) Poor aqueous solubility and/or propensity of phospholipids and sterols to form membranous or micellar structures at very low, often nanomolar concentrations; (ii) Inability to obtain saturation binding of a putative SCP-2 ligand binding site with sterols or phospholipids *in vitro* (10,14,15,18,30,31); (iii) The use of organic solvents as a vehicle for the lipid ligands. Organic solvents such as ethanol and propylene glycol may interfere with the binding of ligands to SCP-2 (32); (iv) Loss of putative ligand(s) during the SCP-2 purification procedure. It was reported that SCP-2 purified from tissues has no bound ligand (33,34).

Recently, it was shown that SCP-2 has a fatty-acid binding site (32,35). The present investigation explores the acyl specificity and location of the SCP-2 fatty-acid binding site by use of a fluorescent displacement assay and fluorescence energy transfer from the SCP-2 intrinsic aromatic amino acid residue (Trp^{50}) to bound *cis*-parinaric acid (9*Z*,11*E*,13*E*,15*Z*-octadecatetraenoic acid) serving as an energy transfer acceptor. This study takes advantage of the recent observation that *cis*-parinaric acid binds to SCP-2 with saturation kinetics (32,35) and

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Abbreviations: SCP-2, sterol carrier protein-2; L-FABP, liver fatty-acid binding protein; NMR, nuclear magnetic resonance; *cis*-parinaric acid, 9*Z*,11*E*,13*E*,15*Z*-octadecatetraenoic acid.

the presence of a single aromatic amino acid residue, Trp^{50}, in SCP-2 (8).

MATERIALS AND METHODS

Materials. Cis-parinaric acid was obtained from Molecular Probes (Eugene, OR). *Cis*-parinaroyl CoA was synthesized and purified as described earlier (36). Dimethylallyl pyrophosphate, farnesyl pyrophosphate, geranyl pyrophosphate, geranyl geranyl pyrophospate, CoA (CoASH), phytanic acid, acyl-CoA synthase, and ATP were from Sigma Chemical Co. (St. Louis, MO). Saturated and unsaturated fatty acids were obtained from Nu-Chek-Prep Inc. (Elysian, MN). Phytenic acid was kindly provided by Drs. K. Tomer and Cary Weinberger (National Institutes of Health, Research Triangle Park, NC). Pristanic acid was a generous gift from Dr. A. Moser (Johns Hopkins University, Baltimore, MD). L-Tryptophan was from Calbiochem (San Diego, CA). All other chemicals were reagent grade or better.

Methods. Recombinant human SCP-2 and native rat liver fatty-acid binding protein (L-FABP) were isolated and purified as described earlier (37,38). L-FABP was delipidated as described (39). Protein purity was >99% as determined by silver-stained SDS polyacrylamide gel electrophoresis. SCP-2 and L-FABP concentration were determined by Bradford assay (40) (Bio-Rad Laboratories, Richmond, CA). L-FABP concentration determined by the Bradford assay was corrected according to amino acid analysis (41). The Bradford assay overestimates the L-FABP concentration by 1.69-fold.

Absorption and steady-state fluorescence spectroscopy. Absorption spectra were measured at room temperature (~24°C) using a UV/VIS Lambda 2 Double-Beam Spectrophotometer (Perkin-Elmer Inc., Norwalk, CT). Corrected (unless otherwise noted) steady-state fluorescence spectra were measured with an ISS PC1 photon counting spectrofluorimeter (ISS Instruments, Champaign, IL) in a quartz fluorescence cuvette, optical path length 1 cm. Temperature was maintained at 25°C (±0.1°C) in a thermostated cell holder. The excitation and emission bandwidths were 4 and 8 nm, respectively.

Displacement of cis*-parinaric acid bound to SCP-2 or L-FABP by fatty acids*. SCP-2 (0.18 μM) or L-FABP (0.10 μM) in 25 mM phosphate buffer (pH 7.4) were incubated with 0.44 and 0.30 μM *cis*-parinaric acid, respectively, for 5 min at 25°C to obtain stable fluorescence. Protein-bound *cis*-parinaric acid was displaced by fatty acids (3.0–4.5 μM) with acyl chain lengths ranging from 10 to 20 carbons. After addition of displacing fatty acid, the sample was allowed to equilibrate for 5 min before measurement of *cis*-parinaric acid fluorescence.

The form in which the fatty acids were added to the incubation mixture was important owing to the relatively low solubility of long-chain fatty acids in aqueous buffer. The critical micellar concentration of long-chain fatty acids is in the micromolar range (42,43). Thus, displacement by a very soluble medium-chain fatty acid (C_{10}) would not be complicated by the presence of micellar equilibria while, in contrast, the displacement by the long-chain fatty acid (C_{20}) would be complicated by multiple equilibria reflecting the affinities of both the micelle and the SCP-2 for the fatty acid. To minimize these complications related to the differential solubility of the displacing fatty acids, all displacing fatty acids were used as their sodium salts. The critical micellar concentration for the C_{10}–C_{20} fatty acid salts is 1000-fold higher, ~ several millimolar (44), as compared to the corresponding free fatty acids. Fatty acid sodium salts were prepared by dissolving free fatty acids in 10 mM NaOH. It should be noted that fatty acids with chain lengths shorter than C_{10} did not displace bound ligands (data not shown). Measurements were corrected for the blank (ligand or protein only) and for photobleaching (<1%).

Fluorescence quantum yield. Fluorescence quantum yield of SCP-2 in phosphate buffer (ϕ_{prot}) was measured by a relative method (45) as:

$$\phi_{prot} = \phi_{ref}\left[\frac{1-10^{-D_{ref}}}{1-10^{-D_{prot}}}\right]\left(\frac{S_{prot}}{S_{ref}}\right)\left(\frac{n_{prot}}{n_{ref}}\right)^2 \quad [1]$$

where ϕ_{prot} and ϕ_{ref} are the protein and the reference fluorescence quantum yield, respectively; S_{prot} and S_{ref} are integral emission of the protein and the reference, respectively; D_{ref} and D_{prot} are optical densities at excitation wavelength for the reference and the protein, respectively; and n_{prot} and n_{ref} are the respective protein and reference refractive indices. L-Tryptophan in phosphate buffered saline was used as the reference, $\phi_{ref} = 0.14$ at 25° (46). The value of n_{prot} was assumed to be 1.4 (47). The relative error in the measurement of ϕ_{prot} was ≤0.02.

RESULTS

Fatty-acid specificity of the SCP-2 and L-FABP fatty-acid binding sites. Cis-parinaric acid is a naturally occurring 18-carbon, kinked-chain, fluorescent analog of oleic acid that fluoresces poorly in aqueous solution, possibly owing to strong electrostatic interactions of this fluorophore with water molecules. When bound to SCP-2 or L-FABP, *cis*-parinaric acid fluoresces strongly, and Scatchard analysis indicates this fatty acid binds to SCP-2 and L-FABP with 1:1 and 2:1 stoichiometry, respectively. The respective binding affinities were estimated to be $K_d = 0.18$ and 0.41 μM, respectively (32,35).

The *cis*-parinaric acid binding assay was adapted to examine fatty-acid specificity of SCP-2 binding site *via* displacement of bound *cis*-parinaric acid. As shown in Figure 1, SCP-2 bound *cis*-parinaric acid was displaced by a variety of 10–22 carbon length fatty acids. However, this displacement ability was highly dependent on the chain length and degree of saturation of the various fatty acids. Among the saturated fatty acids, the C_{14} and C_{16} fatty acids were the most efficient displacers while shorter- or longer-chain fatty acids were very poor displacers (Fig. 1A). The monounsaturated fatty acids of equal chain length had approximately similar ability as the corresponding saturated fatty acids to displace SCP-2 bound *cis*-parinaric acid (Fig. 1B vs. 1A). In contrast, the polyunsat-

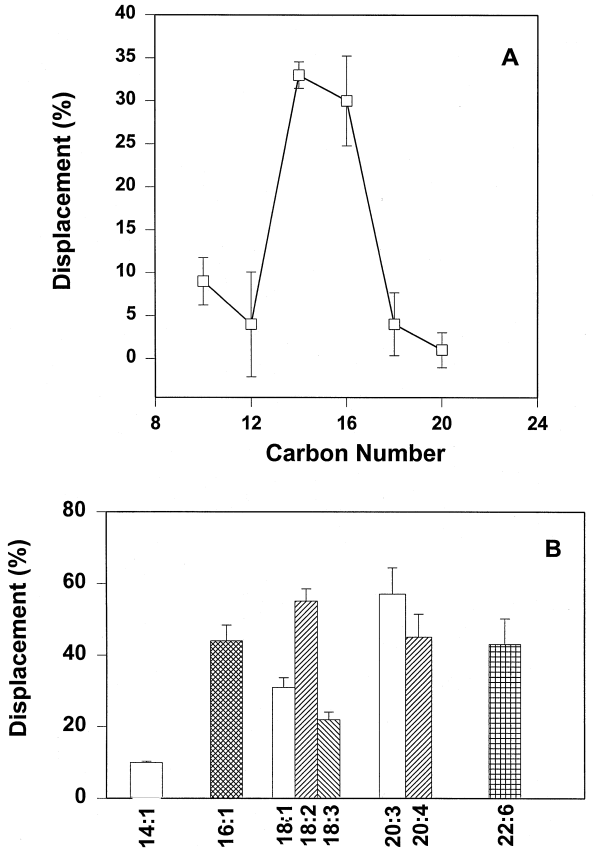

FIG. 1. Displacement of sterol carrier protein-2 (SCP-2) bound *cis*-parinaric acid by fatty acids. SCP-2 (0.18 μM) was preincubated with *cis*-parinaric acid (0.44 μM) followed by addition of displacing fatty acid (4.5 μM) as described in the Methods section. Values represent the mean ± SE (n = 3).

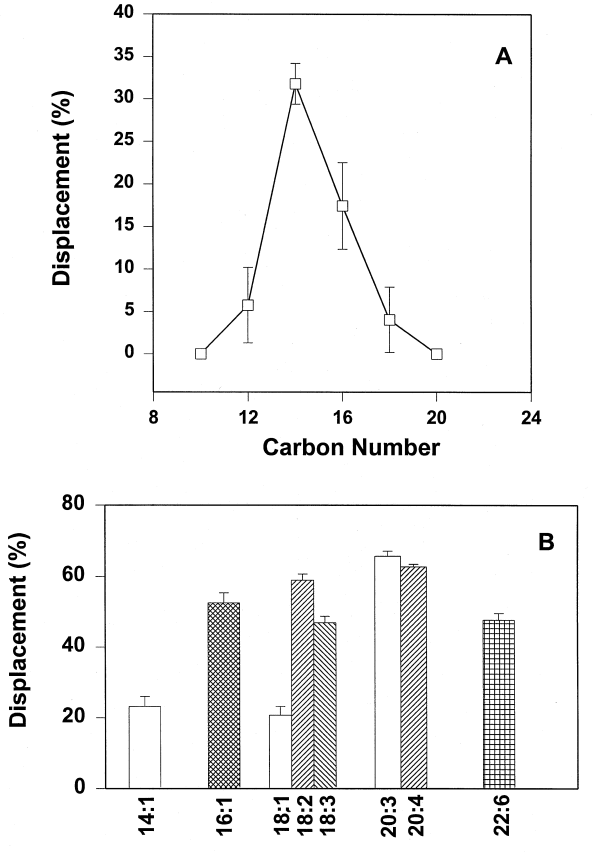

FIG. 2. Displacement of liver fatty-acid binding protein (L-FABP) bound *cis*-parinaric acid by fatty acids. L-FABP(0.1 μM) was preincubated with *cis*-parinaric acid (0.3 μM) followed by addition of displacing fatty acid (3 μM) as described in the Methods section. Values represent the mean ± SE (n = 3).

urated fatty acids were in general much more efficient displacers than the corresponding chain length saturated fatty acids (Fig. 1B vs. 1A).

The *cis*-parinaric acid binding assay was also applied to examine fatty-acid specificity of L-FABP *via* displacement of bound *cis*-parinaric acid. As shown in Figure 2, L-FABP bound *cis*-parinaric acid was readily displaced by a variety of 12–22 carbon length fatty acids. This displacement ability was also highly dependent on the chain length and degree of saturation of the various fatty acids. Among the saturated fatty acids, the C_{14} and C_{16} fatty acids were the most efficient displacers while shorter- or longer-chain fatty acids were very poor displacers (Fig. 2A). This pattern was very similar to that observed for SCP-2 (Fig. 1A). The monounsaturated fatty acids of equal chain length had approximately equal or similar ability as the corresponding saturated fatty acids to displace L-FABP bound *cis*-parinaric acid (Fig. 2B vs. 2A). In contrast and as observed for SCP-2, the polyunsaturated fatty acids were, in general, much more efficient displacers of L-FABP-bound *cis*-parinaric acid than the corresponding chain length saturated fatty acid (Fig. 1B vs. 1A).

Interaction of isoprenoids with the SCP-2 and L-FABP fatty-acid binding site. The *cis*-parinaric acid displacement assay was applied to further examine the specificity of SCP-2 and L-FABP fatty-acid binding sites for other hydrophobic molecules such as isoprenoids. Isoprenoids such as dimethylallyl-PP (C_5), farnesyl-PP (C_{10}), geranyl-PP (C_{15}), and geranyl geranyl-PP (C_{20}) are branched-chain aliphatic molecules that are intermediates in the microsomal and/or peroxisomal synthesis of cholesterol, dolichol, and ubiquinone. They are basically comprised of isopentyl units with increasing chain length. As shown in Table 1, the pyrophosphate derivatives of C_5 to C_{20} isoprenoids displaced SCP-2 and L-FABP bound *cis*-parinaric acid. Unlike the fatty acid displacers (Figs. 1 and 2), short-chain isoprenyl pyrophosphates efficiently displaced SCP-2 bound, and less so L-FABP bound, *cis*-parinaric acid (Table 1). In both cases the geranyl geranyl pyrophosphate was a more efficient displacer than the shorter-chain isoprenyl pyrophosphates. Surprisingly, isoprenyl pyrophosphates with chain length from 4–12 were much more efficient in displacing SCP-2 than L-FABP bound *cis*-parinaric acid (Table 1). However, the longest chain length C_{16} geranyl geranyl pyrophosphate was equally effective in displacing SCP-2 and L-FABP bound *cis*-parinaric acid (Table 1). These data suggest that both SCP-2 and L-FABP may play different role(s) in cellular metabolism of isoprenoids.

TABLE 1
Displacement of *cis*-Parinaric Acid Bound to Fatty Acid-Binding Proteins by Isoprenoids[a]

	Displacement (%)	
Displacing agent	SCP-2	L-FABP
Dimethylallyl pyrophosphate	34 ± 2.3	8 ± 0.9
Farnesyl pyrophosphate	40 ± 1.7	17 ± 2.1
Geranyl pyrophosphate	34 ± 2.1	13 ± 1.4
Geranyl geranyl pyrophosphate	65 ± 1.2	68 ± 0.9

[a]Sterol carrier protein-2 (SCP-2) and liver fatty-acid binding protein (L-FABP) (0.18 μM) were preincubated with *cis*-parinaric acid (0.45 μM) followed by the addition of displacing ligands (4.5 μM). Values represent mean ± SEM (n = 3–5).

Interaction of branched-chain fatty acids with the SCP-2 and L-FABP fatty acid-binding site. The *cis*-parinaric acid displacement assay was applied to examine if SCP-2 and L-FABP also interacted with branched-chain fatty acids such as those derived from phytol. In ruminants, the phytol side-chain of chlorophyll is cleaved to release phytol which is oxidized to phytanic acid by enteric bacteria such that both the phytanic acid and its precursor phytol are present in human diets containing ruminant fats and dairy products (48). Daily human consumption of phytanic acid is 50–100 mg (49). Phytanic acid is absorbed, transported to the liver, and metabolized (oxidized) in peroxisomes (50). Normal human serum levels of phytanic and phytenic acid are 6 and 2 μM, respectively (51). The C_{16} carbon phytol-derived fatty acids displaced SCP-2 bound, more so than L-FABP, bound *cis*-parinaric acid (Table 2). Phytenic acid displaced both SCP-2 and L-FABP bound *cis*-parinaric acid to the same degree, 22 and 26%, respectively (Table 2). In contrast, phytanic acid and pristanic acid were more effective in displacing L-FABP than SCP-2 bound *cis*-parinaric acid (Table 2). These data suggest that both SCP-2 and L-FABP may play different role(s) in branched-chain fatty-acid metabolism within the cell.

Location of SCP-2 fatty-acid binding site as determined by fluorescence energy transfer from SCP-2 to the bound cis*-parinaric acid*. Although the structure of the L-FABP fatty-acid binding sites was recently reported by X-ray crystallography (52), similar progress has not yet been made with regard to the SCP-2 fatty-acid binding site. The nucleotide sequence of SCP-2 indicates the presence of a single Trp at position 50 and no tyrosine residues in the SCP-2 polypeptide chain (28). Hence, the Trp^{50} may serve as an internal "ruler" or reference point for locating the putative fatty-acid binding site in SCP-2. The proximity of SCP-2 Trp^{50} to the fatty-acid binding site can be determined by fluorescence energy transfer from Trp^{50} to a bound *cis*-parinaric acid. The absorbance spectrum of the SCP-2 bound *cis*-parinaric acid was determined at a fatty acid to protein molar ratio of 1:10, which corresponds to approximately 90% of the fatty acid bound to SCP-2. The unbound free *cis*-parinaric acid poorly absorbs in aqueous buffers owing to strong electrostatic interactions with water molecules. These factors allowed determination of the absorption spectrum of SCP-2 bound *cis*-parinaric acid by measuring the differential spectrum with SCP-2/*cis*-parinaric acid in the sample and SCP-2 in the reference. This explains why the protein absorption component is not shown in Figure 3. The absorbance spectrum of *cis*-parinaric acid bound SCP-2 displayed maxima near 292, 306, and 320 nm (Fig. 3, dashed line). The nonzero absorbance of *cis*-parinaric acid at 250 nm was due to nonactive absorbance of the aqueous buffer which was very difficult to compensate owing to strong absorption of water in that spectral region.

Upon excitation at 310 nm, a nonstructured emission spectrum was observed with a peak at ~416 nm (data not shown). As shown in Figure 3 (solid line), the SCP-2 Trp^{50} fluorescence emission spectrum significantly overlaps the absorption spectrum of *cis*-parinaric acid. This provides the resonance conditions required for remote dipole-dipole electron excitation energy transfer (Forster energy transfer) from the SCP-2 Trp^{50} (donor) to the bound *cis*-parinaric acid (acceptor). Forster energy transfer from Trp to *cis*-parinaric acid has been previously used to measure interatomic distances in intestinal FABP (53). The efficiency of Forster energy transfer from SCP-2 Trp^{50} to *cis*-parinaric acid was examined in experiments where SCP-2 was excited at 255 nm, and SCP-2 Trp^{50} fluorescence emission at 332 nm was monitored upon titration of SCP-2 with increasing amounts of *cis*-parinaric

TABLE 2
Displacement of *cis*-Parinaric Acid Bound to Fatty Acid-Binding Proteins by Phytol Derivatives[a]

	Displacement (%)	
Displacing agent	SCP-2	L-FABP
Phytenic acid	22 ± 0.6	26 ± 0.7
Phytanic acid	28 ± 2.6	48 ± 1.0
Pristanic acid	26 ± 2.4	48 ± 0.9

[a]SCP-2 and L-FABP (0.18 μM) were preincubated with *cis*-parinaric acid (0.45 μM) followed by the addition of displacing ligands (4.5 μM). Values represent mean ± SEM (n = 3–5). See Table 1 for abbreviations.

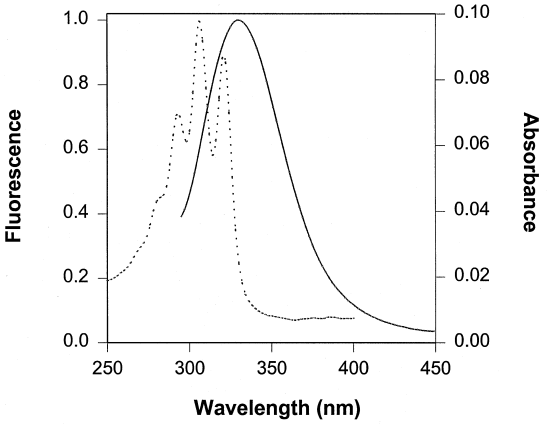

FIG. 3. Spectral characteristics of SCP-2 and *cis*-parinaric acid. Fluorescence emission spectra of SCP-2 (1 μM) in phosphate buffer, pH 7.4 (excitation at 280 nm, solid line). Absorption spectrum of *cis*-parinaric acid (1.3 μM) bound to SCP-2 (dotted line). See Figure 1 for abbreviation.

acid acceptor (Fig. 4A). SCP-2 Trp^{50} was excited at 255 nm because at this wavelength the acceptor, *cis*-parinaric acid, had negligible absorbance over the concentration range used in the titration experiments. As shown, upon titration of SCP-2 with increasing amounts of *cis*-parinaric acid, the tryptophanyl emission of SCP-2 gradually declined to ~40% of its initial level (legend to Fig. 4A). Such gradual decrease of tryptophanyl emission was accompanied by the appearance of an additional fluorescence emission band near ~420 nm (Fig. 4B) which can be attributed to the sensitized emission of *cis*-parinaric acid. It should be noted that no detectable SCP-2 fluorescence quenching was observed when the protein emission was monitored during SCP-2 titration with nonfluorescent oleic acid (data not shown). This observation strongly suggests that SCP-2 tryptophanyl fluorescence quenching detected in the presence of fluorescent *cis*-parinaric acid cannot be explained by direct interaction of two fluorophores, or by conformational changes of the protein induced by a bound fatty acid. Therefore, taken together, the above observations regarding the SCP-2 fluorescence quenching in the presence of *cis*-parinaric acid are consistent with the predominant mechanism of this quenching process being fluorescence energy transfer from SCP-2 Trp^{50} to the bound *cis*-parinaric acid. The efficiency of SCP-2 fluorescence quenching by *cis*-parinaric acid was estimated as 60% (Fig. 4A). Based on this energy transfer efficiency, the critical distance of energy transfer, R_o, for the SCP-2 Trp^{50} and *cis*-parinaric acid donor/acceptor pair was calculated as (54):

$$R_o = (9.765 \times 10^3)(K^2JQn^{-4})^{1/6} \quad [2]$$

where Q is the quantum yield of the donor in the absence of the acceptor; n, the refractive index; J, the overlap integral is defined as:

$$J = \int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \Big/ \int F_D(\lambda)d\lambda \quad [3]$$

where $F_D(\lambda)$is the normalized emission spectrum of the donor; $\varepsilon_A(\lambda)$ is the absorption spectrum of acceptor. Finally K^2 is the orientation factor ranging from 0 to 4. Energy transfer efficiency, E, is related to the distance between the donor and the acceptor r by:

$$E = R_o^6 \Big/ (R_o^6 + r^6) \quad [4]$$

The values of the parameters describing the Forster energy transfer in the SCP-2 Trp^{50}/*cis*-parinaric acid donor/acceptor pair are presented in Table 3. The calculated intermolecular distance between SCP-2 Trp^{50} and the *cis*-parinaric acid, based on the assumption that $K^2 = 2/3$ (i.e., for random orientation of the donor and the acceptor moments) was $r = 40$ Å (Table 3).

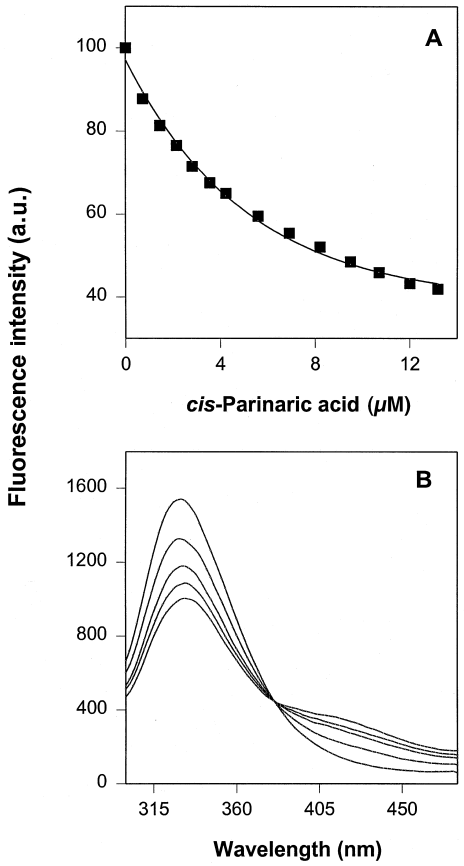

FIG. 4. Forster energy transfer between SCP-2 tryptophanyl (the donor) and bound *cis*-parinaric acid (the acceptor). (A) SCP-2 (1 μM) fluorescence quenching upon its titration with fatty acid. Excitation at 255 nm, emission at 332 nm. (B) Fluorescence emission spectra of SCP-2 (1 μM) in the presence of increasing amounts of *cis*-parinaric acid. From top to bottom: 1 μM SCP-2; 1 μM SCP + 0.5 *cis*-parinaric acid; 1 μM SCP + 1.0 μM *cis*-parinaric acid; 1 μM SCP + 1.5 μM *cis*-parinaric acid; 1 μM SCP + 2 μM *cis*-parinaric acid; 1 μM SCP + 2.5 μM *cis*-parinaric acid. Excitation at 255 nm. See Figure 1 for abbreviation.

TABLE 3
Forster Energy Transfer Parameters for Fluorescence Energy Transfer from SCP-2 Trp^{50} to Bound *cis*-Parinaric Acid (PA)[a]

Donor/acceptor	J (M^{-1} cm^3)	R_o[b] (Å)	E (%)	$r_{2/3}$[c] (Å)
Trp/*cis*-PA	9.82 10^{-15}	43	60	40

[a]J, overlap integral; R_o, critical distance at which 50% energy transfer occurs; E, energy transfer efficiency; $r_{2/3}$, actual distance between two residues, with the assumption $K^2 = 2/3$ (where K^2 is the orientation factor).
[b]Calculated for $\phi_{prot} = 0.27$; $n = 1.4$, and $K^2 = 2/3$, where ϕ_{prot} is quantum yield and n is refractive index.
[c]Calculated for $K^2 = 2/3$. See Table 1 for other abbreviation.

DISCUSSION

Liver hepatocytes and intestinal enterocytes both contain SCP-2 and L-FABP (reviewed in Ref. 55). The physiological function of neither protein is known. Therefore, a knowledge on the ligand binding specificities of these proteins is important to begin understanding their potential functional role(s). While SCP-2 is known to enhance the intermembrane transfer of cholesterol, glycolipids and phospholipids, identification of a ligand binding site has been problematic owing to the poor aqueous solubility of the ligands involved as well as their high affinity for membranes (8,9,18,56,57).

Only recently was it recognized that SCP-2 binds fatty acids (32,58). Specific binding of fatty acids was previously not detected with the standard Lipidex 1000 (Sigma Chemical Co.) radioligand competition binding assay, because of the sensitivity of the SCP-2 fatty-acid binding site to the organic solvent vehicle used in this competition assay (32,59). In contrast, the level of organic solvent used in the Lipidex 1000 assay did not interfere with fatty-acid binding to L-FABP (32,59). Furthermore, unlike the L-FABP whose X-ray crystal structure with bound fatty acid is now available (52), little is known regarding the specificity of SCP-2 for long-chain aliphatic molecules or the location of the SCP-2 fatty-acid binding site. Although previous studies have examined the fatty-acid specificity of L-FABP, the respective ligands were generally used as the free fatty acids rather than the fatty-acid salts (reviewed in Refs. 2,43,60–62). As pointed out previously (11) and in the introductory section, this may provide an apparent fatty-acid binding ability complicated by the solubility of the fatty acid.

The present investigation examined for the first time the specificity of the fatty-acid binding site of SCP-2 and L-FABP by use of sodium salts of the fatty acids. In a displacement assay involving protein-bound fluorescent *cis*-parinaric acid, a series of unbranched saturated and unsaturated fatty acids, isoprenoids, as well as branched-chain fatty acids were examined. The data demonstrated that SCP-2 bound a variety of fatty acids with a similar chain length and unsaturation specificity pattern as did L-FABP. Furthermore, both proteins bound a series of isoprenyl pyrophosphates and branched-chain fatty acids derived from phytol. These ligands were able to displace SCP-2 and L-FABP bound *cis*-parinaric acid to similar extents as did the normal fatty acids. However, the two proteins exhibited distinct differences in their binding of these branched aliphatic chain ligands. It should be noted that the displacement assay does not measure absolute binding affinities, but rather utilizes a fixed set of conditions to compare the relative ability of a series of lipophilic molecules to displace SCP-2 bound *cis*-parinaric acid. Conclusions regarding the absolute values of the respective ligand affinities cannot necessarily be made from displacement assays. This has been shown for fatty-acid vs. fatty acyl-CoA binding to SCP-2, wherein the binding affinity of SCP-2 for these ligands differs two orders of magnitude (35). However, when the displacement conditions were optimized for the lower-affinity ligand, fatty acid, the relative displacement of fluorescent fatty acid by both fatty acid and fatty acyl-CoA did not reflect the absolute differences in K_ds (35).

The significance of the data obtained with regard to SCP-2, which has been localized to peroxisomes (both interior and cytoplasmic surface) and to a lesser extent other organelles such as mitochondria and endoplasmic reticulum (5,6), is several-fold: First, SCP-2 may enhance import of fatty acids and/or fatty acyl-CoA into peroxisomes or mitochondria and thereby stimulate fatty-acid β-oxidation. Second, by binding phytol-derived fatty acids, SCP-2 may enhance import of branched-chain fatty acids into peroxisomes for α-oxidation. Third, there is growing evidence that peroxisomes contain most of the enzymes for cholesterol biosynthesis (63). Therefore, by binding isoprenyl pyrophosphates, SCP-2 may be involved in peroxisomal as well as microsomal cholesterol biosynthesis. Fourth, by binding a variety of short- and long-chain isprenyl pyrophosphates, SCP-2 may influence dolichol biosynthesis. Dolichol is an important molecule involved in glycosylation of proteins, and SCP-2 has already been shown to enhance the activity of microsomal *cis*-prenyltransferase (64). Fifth, experiments with transfected L-cell fibroblasts expressing SCP-2 have shown that the microsomal esterification of cholesterol is enhanced (65). Thus, by exhibiting fatty acyl chain selectivity, SCP-2 may be involved in the subsequent utilization of fatty acyl-CoA for microsomal esterification of fatty acids to cholesteryl esters. Similar considerations for L-FABP must be modified by its preferential location in cell cytosol and in the region of the endoplasmic reticulum (reviewed in Ref. 66). In summary, the interaction of SCP-2 as well as L-FABP with a wide variety of aliphatic molecules (branched-chain fatty acids, and isoprenoids) as well as normal fatty acids suggests new potential roles for these proteins in lipid metabolism.

Although SCP-2 stimulates the intermembrane transfer of many different lipids, including sterols, phospholipids, glycolipids, etc., it is unclear whether these activities actually require a ligand binding site in the protein. As a basic protein SCP-2 may, by binding to the anionic surface of membranes, disrupt membrane structure and thereby enhance the desorption of lipid molecules from the membrane. Recent findings, however, indicate that this is not likely for SCP-2 (23). In order to function in intermembrane sterol transfer, for example, SCP-2 must bind sterol (23). The location of this sterol binding site has not been determined. Neither is it known whether any of the other lipids transferred by SCP-2 also bind to the same or different sites in SCP-2.

The first major step in resolving a putative SCP-2 ligand binding site was the determination of the tertiary structure of the apo SCP-2. Based on NMR data, it was concluded that apo SCP-2 is comprised of a polypeptide chain with three α-helixes, a five-stranded β-sheet, with the rest as turn or random coil (29). SCP-2 Trp^{50} is located on one side of the SCP-2 molecule in the middle of β-sheet II. However, the orientation (facing the aqueous or the hydrophobic interior) of the tryptophanyl aromatic ring in β-sheet II is not known. On the opposite side of the molecule is located β-sheet V, representing the C-terminal β-sheet, to which is attached a randomly oriented "tail." The NMR data further show that the overall shape of apo SCP-2 is roughly spherical or slightly ellipsoidal (29). This is in agreement with recently presented data that the rotational correlation time and hydrodynamic radius of the entire SCP-2 protein are consistent with a nearly spherical or slightly elliptical protein (35). The apo SCP-2 Trp^{50} fluorescence dynamics showed an overall protein rotational correlation time of 8.4 ns and a hydrodynamic radius of 20 Å, which is very similar to that of the theoretical radius (18 Å) of a 13.2 kDa (the molecular weight of apo SCP-2)

globular protein. This was also confirmed by the measurements of the rotational dynamics of SCP-2 bound *cis*-parinaric acid, which yielded a hydrodynamic radius of 20.5 Å (35). These data indicate that SCP-2 is a globular, slightly elongated protein. In contrast, an early fluorescence study of apo SCP-2 Trp^{50} fluorescence dynamics measured a much longer rotational correlation time of 13.8 ns and suggested that apo SCP-2 is highly elliptical with an axial ratio of nearly 3:1 (31). The reason for the discrepancy between the latter study vs. the NMR and the present results is not clear. However, in the earlier work, the longer 13.8 ns rotational relaxation time was obtained at high SCP-2 concentration, 10 μM (31), and suggests the presence of SCP-2 dimers. Indeed, at concentrations of 10 μM (instead of the 0.18 μM used in the present work), we have observed a similar longer rotational relaxation time near 15 ns, indicative of SCP-2 dimers (data not shown).

The second major contribution to resolving the location of an SCP-2 ligand binding site came from results of site-directed mutagenesis (67). As mentioned above, the apo SCP-2 polypeptide chain is comprised of three α-helixes and a five-stranded β-sheet, one of which (β-sheet V) is on the opposite side of the SCP-2 Trp^{50} (located in β-sheet II) (29). Site-directed mutagenesis indicates that deletion and/or mutations in β-sheet V abolish SCP-2 transfer activity of sterols and phospholipids. Two other parts of the SCP-2 molecule (helix A and amino acids next to Cys^{71}) are very near the β-sheet V and are also necessary for sterol and phospholipid transfer activity (29). Although the site-directed mutagenesis data do not provide definitive proof, they suggest that a sterol and/or phospholipid binding site may be in a cleft formed by β-sheet V, a portion of helix A, and amino acids around Cys^{71}.

The present results show, for the first time, direct data on the location of the fatty-acid binding site in holo SCP-2. Earlier results from this laboratory showed that SCP-2 binds fluorescent fatty acids with saturation kinetics and high affinity, K_ds 0.2–0.4 μM (32,35). Nevertheless, the location of the SCP-2 fatty-acid binding site and/or its relation to putative cholesterol and/or phospholipid binding/transfer site(s) were not known. The data presented herein show that SCP-2 Trp^{50} is able to transfer energy to bound *cis*-parinaric acid with high efficiency near 60%. This tryptophan was not exposed to aqueous solvent, as shown by the emission maximum wavelength (λ = 332 nm), and by the lack of oleic acid effect on the Trp^{50} emission intensity/spectrum. Therefore, the aromatic ring of Trp^{50}, located on β-sheet II, appears to be turned inward to the hydrophobic core of the protein. Forster energy transfer showed that the estimated distance between SCP-2 Trp^{50} and bound *cis*-parinaric acid was near 40 Å, based on an assumed random orientation of the donor and the acceptor. The experimentally determined and theoretically calculated diameters for apo SCP-2 were 41 and 37.6 Å, respectively (35). The experimentally determined diameter for holo-SCP-2 containing bound oleic acid was 49 Å (35). It should be noted that the orientation of the fatty acid in the SCP-2 binding site is with the carboxylate exposed near the surface (35). An examination of the structure of *cis*-parinaric acid indicates that the tetraene fluorophore extends from the middle of the molecule to its methyl terminal of the *cis*-parinaric acid, i.e., about 8–10 Å away from the carboxyl terminus (35). This would suggest that the assumed orientation factor used to calculate the distance between SCP-2 Trp^{50} and bound *cis*-parinaric acid, 40 Å, was reasonable. More importantly, an intermolecular distance of 40 Å betweeen Trp^{50} and the fluorophore of *cis*-parinaric acid would place the fatty-acid binding site of SCP-2 on the opposite side of the SCP-2 from the β-sheet II Trp^{50}.

In summary, SCP-2 interacted with a wide variety of aliphatic ligands: C_{12} to C_{14} fatty acids, C_{16} branched-chain fatty acids, and C_4 to C_{16} isoprenyl pyrophosphates. The SCP-2 fatty-acid binding site was located approximately 40 Å from (and on the opposite side of) the location of SCP-2 Trp^{50}. The latter observation is consistent with site-directed mutagenesis studies (67), indicating that the β-sheet V, opposite from Trp^{50}, is essential for sterol and phospholipid transfer activity. One interpretation of these facts is that the ligand binding site that interacts with fatty acids, sterols, and phospholipids may be the same site. These ligand binding data may provide new insights into the physiological importance of SCP-2 in the cell.

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Elution Factors of Synthetic Oxotriacylglycerols as an Aid in Identification of Peroxidized Natural Triacylglycerols by Reverse-Phase High-Performance Liquid Chromatography with Electrospray Mass Spectrometry

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ABSTRACT: Selected elution factors were determined for model oxotriacylglycerols as an aid in identification of the peroxidation products of natural triacylglycerols by reverse-phase high-performance liquid chromatography (HPLC) with electrospray mass spectrometry (LC/ES/MS). For this purpose synthetic triacylglycerols of known structure were converted to hydroperoxides, hydroxides, epoxides, and core aldehydes and their dinitrophenylhydrazones by published procedures. The oxotriacylglycerols were resolved by normal-phase thin-layer chromatography and reverse-phase HPLC, and the identities of the oxotriacylglycerols confirmed by LC/ES/MS. Elution factors of oxotriacylglycerols were determined in relation to a homologous series of saturated triacylglycerols, ranging from 24 to 54 acyl carbons, and analyzed by reverse-phase HPLC, using a gradient of 20–80% isopropanol in methanol as eluting solvent and an evaporative light-scattering detector. It was shown that the elution times varied with the nature of the functional group and its regiolocation in the triacylglycerol molecule. A total of 31 incremental elution factors were calculated from chromatography of 33 oxygenated and nonoxygenated triacylglycerol species, ranging in carbon number from 36 to 54 and in double-bond number from 0 to 6.

Lipids 32, 1211–1218 (1997).

High-performance liquid chromatography (HPLC) is widely used to analyze natural triacylglycerols, and numerous authors have noted their orderly elution under isocratic (1–4) and solvent gradient (5–7) elution conditions. Although the elution characteristics of triacylglycerols with functional groups also have been determined (1,8–14), only a few systematic studies have been reported (11–14). Mixtures of oxygenated natural triacylglycerols are difficult to analyze by chromatographic methods because of the presence of a large variety of homologs and of regio- and geometric isomers, which tend to overlap with each other and with homologs of unoxidized parent compounds. In principle, it should be possible to identify and quantify overlapping species by combining HPLC with on-line mass spectrometry (MS), which can provide mass-to-charge (*m/z*) ratios for both molecular and fragment ions characteristic of particular triacylglycerols. The thermospray (13,15) and chemical ionization (5,15) routines employed for this purpose, however, are not entirely satisfactory for analysis of the unstable hydroperoxides of triacylglycerols. In contrast, the milder electrospray ionization yields the molecular ion as the major or sole species for each triacylglycerol (16) or its hydroperoxide, epoxide, and core aldehyde (17,18), and usually does not yield fragment ions, unless the ionization voltage is greatly increased (15). Furthermore, many oxotriacylglycerols possess molecular masses which match the various ordinary triacylglycerols, or their M + 1 or M + 2 ions. In order to provide a rationale for identification of overlapping and unknown triacylglycerols, it is critical to know the relative retention times of the triacylglycerols containing various combinations of oxygenated and nonoxygenated fatty acids. The following study reports the incremental elution factors obtained for model oxotriacylglycerols on reverse-phase HPLC, from which the retention times of unknown oxotriacylglycerols can be estimated by summation and extrapolation, permitting identification of triacylglycerol oxidation products using a single quadrupole liquid chromatography/electrospray/MS (LC/ES/MS) instrument.

MATERIALS AND METHODS

Chemicals and reagents. Tert-butyl hydroperoxide (TBHP) was purchased from Sigma Chemical Co. (St. Louis, MO) and 2,4-dinitrophenylhydrazine (DNPH) and 3-chloroperoxybenzoic acid from Aldrich Chemical Co. (Milwaukee, WI). Methanol (HPLC grade) was obtained from Mallinckrodt

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Abbreviations: DNPH, 2,4-dinitrophenylhydrazine; ECN, equivalent carbon number; ES, electrospray; F_i, correction factor; HPLC, high-performance liquid chromatography; LC, liquid chromatography; M, molecular ion; MS, mass spectrometry; TBHP, *tert*-butyl hydroperoxide; TCN, theoretical carbon number; TLC, thin-layer chromatography; TPP, triphenylphosphine.

Chemicals, Inc. (Paris, KY) and 2-propanol (HPLC grade) from Caledon Laboratories, Ltd. (Georgetown, Ontario, Canada). All other chemicals and solvents were of reagent grade or better quality and were obtained from local suppliers. Synthetic saturated monoacid triacylglycerols were included in HPLC standard (G-1) obtained from Nu-Chek-Prep, Inc. (Elysian, MN). Synthetic 1,3-distearoyl-2-oleoyl and 1,3-dipalmitoyl-2-linoleoyl-*sn*-glycerols were purchased from Sigma Chemical Co. Synthetic 1,2-distearoyl-3-oleoyl, 1-oleoyl-2,3-dipalmitoyl, 1,3-distearoyl-2-oleoyl, 1,2-dioleoyl-3-stearoyl, 1,2-distearoyl-3-linoleoyl, 1,2-dilinoleoyl-3-stearoyl, 1,3-distearoyl-3-linolenoyl, 1,2-dilinolenoyl-3-stearoyl, and 1,2-distearoyl-3-eicosenoyl-*sn*-glycerols were prepared in the laboratory by reacting 1,2-distearoyl-, 3-stearoyl-, or 2,3-dipalmitoyl-*sn*-glycerol with oleoyl, linoleoyl, linolenoyl, and 11-eicosenoyl chlorides in the presence of dry pyridine (19). The 1,2-distearoyl-, 3-stearoyl-, and 2,3-dipalmitoyl-*sn*-glycerols were gifts from Dr. Buchnea. Table 1 lists the synthetic triacylglycerols along with their oxo-derivatives prepared in this study.

Preparation of core aldehydes. Core aldehyde standards were obtained by triphenylphosphine (TPP) reduction of ozonides as previously described (8,17,20). Briefly, 1–40 mg of triacylglycerols were dissolved in 4 mL hexane and ozonized for 5 min in a dry ice/acetone bath. After blowing down the solvent, the ozonides were redissolved in 2–4 mL chloroform and reduced to aldehydes by reacting with 20–40 mg TPP for 1 h at room temperature. The aldehydes were purified by thin-layer chromatography (TLC) as described below. Part of the aldehydes were converted into the DNPH derivatives by treating with 5–10 mL of the DNPH reagent (1 mg DNPH in 2 mL of 1 M HCl) at room temperature overnight (21). The DNPH derivatives were recovered from the solution by chloroform extraction and were further purified by TLC. Specifically, monoaldehydes (Id–e, IId–e) were prepared from monounsaturated triacylglycerols (I–II); see Table 1 for details of name terminology. Dialdehydes (IIId–e) were prepared from triacylglycerols (III, VI–VII) containing two unsaturated fatty acids and one saturated fatty acid.

Preparation of hydroperoxides. Hydroperoxides (Ia, IIa,

TABLE 1
Reference Compounds for Estimation of Elution Factors[a]

Number	Mass[b]	TAG	Number	Mass[b]	Derivatized TAG
I	888	18:0-18:0-18:1	Ia	920	18:0-18:0-18:1 OOH
			Ib	904	18:0-18:0-18:1 OH
			Ic	904	18:0-18:0-18:1 epoxy[c]
			Id	778	18:0-18:0-9:0 ALD
			Ie	958	18:0-18:1-9:0 ALD DNPH
II	888	18:0-18:1-18:0	IIa	920	18:0-18:1-18:0 OOH
			IIb	904	18:0-18:1-18:0 OH
			IIc	904	18:0-18:1-18:0 epoxy[c]
			IId	778	18:0-9:0 ALD-18:0
			IIe	958	18:0-9:0 ALD-18:0 DNPH
III	886	18:1-18:1-18:0	IIIa	950	18:1-18:1-18:0 di-OOH
			IIIb	918	18:1-18:1-18:0 di-OH
			IIIc	918	18:1-18:1-18:0 diepoxy[c]
			IIId	666	9:0ALD-9:0 ALD-18:0[d]
			IIIe	1026	9:0ALD-9:0 ALD-18:0 diDNPH[d]
IV	886	18:0-18:0-18:2	IVa	918	18:0-18:0-18:2 OOH
			IVb	902	18:0-18:0-18:2 OH
			IVc	902	18:0-18:0-18:2 diepoxy[c]
V	830	16:0-18:2-16:0	Va	862	16:0-18:2-16:0 OOH
			Vb	846	16:0-18:2-16:0 OH
VI	882	18:2-18:2-18:0	VIa	914	18:2-18:2-18:0 OOH[e]
			VIb	946	18:2-18:2-18:0 di-OOH
			VIc	898	18:2-18:2-18:0 OH[e]
			VIb	914	18:2-18:2-18:0 di-OH
VII	884	18:0-18:0-18:3			
VIII	878	18:3-18:3-18:0			
IX	916	18:0-18:0-20:1			
X	884	18:1-18:1-18:1			
XI	878	18:2-18:2-18:2			
XII	470–890	Series of saturated TAG (24:0–54:0)[f]			

[a]Abbreviations : ALD, aldehyde; DNPH, dinitrophenylhydroxide; TAG, triacylglycerol.
[b]Nominal mass.
[c]Position of an epoxy group has been marked as an underlined double bond.
[d]These references were also prepared from triacylglycerols VI and VIII.
[e]These compounds were prepared but not used to estimate elution factors.
[f]Reference compounds were co-injected with this series of saturated TAG.

IIIa, VIa) were obtained by oxidizing 0.25–10 mg of a triacylglycerol (I–VI) with 0.5–2.0 mL of 70 mM TBHP for 20 min to 30 h at 22°C or 37°C (13,22). Lipids were extracted with chloroform/methanol (2:1, vol/vol) and the excess TBHP was washed out with water. Some hydroperoxides (IVa, VIa–b) were also prepared by the photosensitized oxidation method described by Neff *et al*. (23). About 2–3 mg triacylglycerol (IV, VI) was added to 3 mL 0.1 mM methylene blue in dichloromethane in a 5-mL beaker on an ice bath, which was placed under a 500 W photographer's lamp.

Preparation of hydroxy triacylglycerols. Hydroperoxides (Ia, IIa, IIIa, IVa, Va, VIa–b) were reduced to the corresponding hydroxytriacylglycerols (Ib, IIb, IIIb, IVb, Vb, VIc–d) by sodium borohydride (24). One mg of hydroperoxide was dissolved in 1 mL of chloroform/methanol (2:1, vol/vol), mixed with 1 mL of 0.1 M $NaBH_4$ in methanol, and allowed to react 1 h at 4°C. The hydroxy compounds were recovered by washing the reaction mixture twice with water, blowing down the solvent, and redissolving the residue as required for subsequent HPLC. Alternatively, the hydroperoxides were reduced with TPP (25). Some 10–11 mg TPP in 0.3–1 mL solvent (hexane) was added to 1–5 mg sample of a hydroperoxy triacylglycerol (IV). The hydroxy derivative (IVa) was purified and TPP was removed by TLC.

Preparation of epoxides. Epoxides (Ic, IIc, IIIc, IVc) were obtained by the method of Deffense (26). A sample of 2–5 mg unsaturated triacylglycerol (I–IV) was reacted with 6–8 mg 3-chloroperoxybenzoic acid in 300–400 μL dichloromethane at room temperature for 1–2 h followed by purification using TLC as described next.

TLC. Normal-phase TLC was used to purify triacylglycerols and their derivatives in a neutral solvent system (27). Briefly, H-plates were prepared in the laboratory, and heptane/isopropyl ether/acetic acid (60:40:4, by vol) solution was used as a mobile eluent. The resolved components were recovered from the silica gel by extraction with chloroform/methanol 2:1 (vol/vol). Extracts were washed with distilled water and dried using Na_2SO_4. Triacylglycerols and their hydroperoxy, hydroxy, and epoxy compounds were detected in ultraviolet light after spraying with 2,7-dichlorofluorescein. Free core aldehydes showed a strong purple color with a Schiff base reagent (27). In the same system, the DNPH derivatives of core aldehydes were seen as yellow bands.

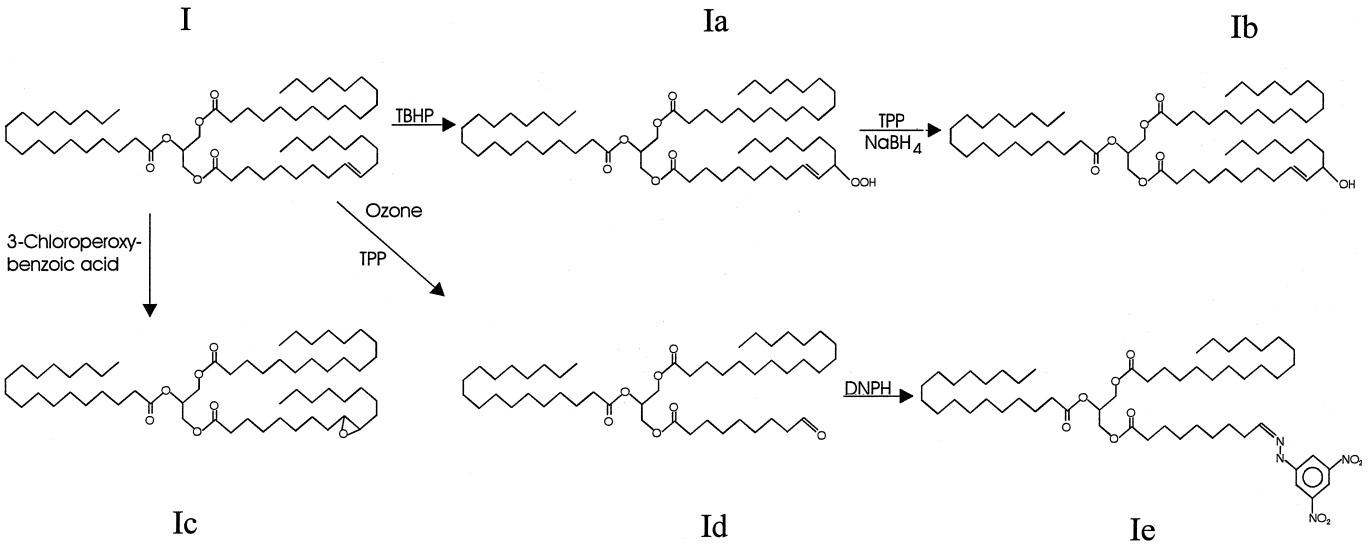
HPLC and LC/ES/MS. Standard oxotriacylglycerols and normal triacylglycerols were resolved by reverse-phase HPLC on a Supelcosil LC-18 column (250 mm × 4.6 mm i.d.; Supelco, Inc., Mississauga, Ontario, Canada) using a linear gradient of 20–80% 2-propanol in methanol (0.85 mL/min) in 30 min (13,17). The instrument was a Hewlett-Packard (Palo Alto, CA) Model 1050 liquid chromatograph, coupled to a Varex ELSD II light-scattering detector (Varex Co., Rockville, MD) using nitrogen as nebulization gas and evaporation temperature of 85°C. The identities of the oxotriacylglycerols were confirmed by LC/MS using a Hewlett-Packard Model 1090 liquid chromatograph interfaced with a nebulizer-assisted ES ionization source connected to a Hewlett-Packard Model 5989A quadrupole mass spectrometer (13,17). The ionization (capillary exit) voltage of this instrument was set at 100 V but could be increased to 300 V, to obtain fragment ions from any clearly resolved components (pseudo MS/MS). The HPLC conditions were the same as just described except that, for ionization, ammonia/isopropanol (1%) was added postcolumn at a flow rate of 0.15 mL/min. The unoxidized and oxidized triacylglycerols were identified as $[M + NH_4]^+$ or $[M + Na]^+$ in positive ion mode, whereas the DNPH derivatives of triacylglycerol core aldehydes were detected as $[M - H]^-$ ions in negative ion mode. Mass spectra were acquired in the 300–1300 mass range over the entire elution profile. The triacylglycerol samples were dissolved in isopropanol (mg/mL) by heating for 2–3 min at 80°C prior to injection. Addition of chloroform to assist dissolution of triacylglycerols was avoided as it caused distortion of peak shape and variation in retention times.

RESULTS AND DISCUSSION

Preparation of reference compounds. The general scheme of preparation of reference compounds is illustrated in Scheme 1 using 1,2-distearoyl-3-oleoyl-*sn*-glycerol as an example. Compounds I to Ie are identified as shown in Table 1. Triacylglycerol (I) was oxidized to a hydroperoxide (Ia) using TBHP. The triacylglycerol hydroperoxide (Ia) was reduced to the hydroxy triacylglycerol (Ib) using TPP. Triacylglycerol (I) was converted into the triacylglycerol core aldehyde (Id) by reaction with ozone followed by reduction of the ozonide intermediate with TPP. The core aldehyde (Id) was converted into the triacylglycerol dinitrophenyl hydrazone (Ic) by reaction with DNPH. The triacylglycerol epoxide (Ic) was prepared by treating triacylglycerol (I) with 3-chloroperoxybenzoic acid. Separate structures are not shown for positional and geometric isomers of the derivatives.

Triacylglycerols containing oleic acid in both primary and secondary positions yielded regioisomers, which could be resolved under appropriate chromatographic conditions. Chromatography also allowed the resolution of the *syn* and *anti* isomers of the DNPH derivatives (see below).

Ozonization has been used for the quantitative preparation of lipid ester core aldehydes (8,17,20). In the present experiments the oleoyl, linoleoyl, and linolenoyl triacylglycerols gave essentially pure 9-oxononanoate esters, which were resolved from the parent triacylglycerols and their ozonides by normal-phase TLC. Upon LC/ES/MS, the free aldehydes showed 32 mass units higher mass than anticipated in both sodiated and ammoniated forms. The increase by 32 mu was attributed to formation of hemiacetal from free aldehyde and methanol, which apparently was formed postcolumn after introduction of ammonia. Substitution of acetonitrile for methanol prevented the formation of the higher mass ions, but aldehydes eluted as broad bands instead of the sharp peaks seen in the isopropanol/methanol system. The mass spectra of the DNPH derivatives of the core aldehydes showed a minor peak with the same mass eluting after the monoaldehyde peak. This



SCHEME 1

peak was attributed to the separation of the *syn* and *anti* isomers. As a result, a total of four isomers of the DNPH derivatives was obtained from a mixture of triacylglycerols containing aldehyde functions in primary and secondary positions of glycerol. Table 2 gives the R_f values of the various oxotriacylglycerols and their derivatization products on normal-phase TLC. The preparation of the triacylglycerol core aldehydes by ozonization and TPP reduction has been discussed recently (17).

Cumene and TBHP have been widely used for the preparation of lipid ester hydroperoxides (28). In the present study, TBHP was added to reference triacylglycerols of known structure to produce the hydroperoxides of oleoyl and linoleoyl triacylglycerols as the main products according to the predictions of Porter *et al.* (29). The 1,3- and 1,2-distearoyl-*sn*-glycerol oleates were more resistant to hydroperoxidation than the corresponding linoleates or the 1,2-dipalmitoyl-*sn*-glycerol linoleate. Some 25–60% of intact monoenes remained after 30–31 h of peroxidation of the oleates compared to 5–30% of intact dienes after only 20–45 min of oxidation of the linoleates. The exact ratio of products to starting materials depended on the ratio of the starting material to the oxidant. In addition to hydroperoxides and original triacylglycerols, the peroxidation mixture also contained other reaction products, which were eluted from the reverse-phase HPLC column between the peaks of the parent triacylglycerols and the hydroperoxides. However, hydroperoxides and residual triacylglycerols were clearly resolved from these compounds. Alternately, photosensitized oxidation (23) provided a fast method to produce sufficient amounts of mono- and dihydroperoxides of 1,2-dilinoleoyl-3-stearoyl. Figure 1 shows the LC/ES/MS profile of a photooxidation mixture

TABLE 2
Average R_f Values of TAG and Oxotriacylglycerols as Obtained on Silica Gel Using a Neutral Lipid Solvent System[a]

Species	R_f value
TAG	0.66 ± 0.04
Monoepoxy	0.48 ± 0.04
Monohydroperoxy	0.46 ± 0.04
Monoaldehyde	0.37 ± 0.04
Monoaldehyde DNPH	0.36 ± 0.04
Monohydroxy	0.36 ± 0.04
Dihydroperoxy	0.34 ± 0.04
Diepoxy[b]	0.34 ± 0.04
Diepoxy[c]	0.29 ± 0.04
Dihydroxy	0.24 ± 0.04
Dialdehyde-DNPH	0.19 ± 0.04
Dialdehyde	0.13 ± 0.04

[a]Silica Gel H60 and heptane/isopropyl ether/acetic acid (60:40:4, by vol).
[b,c]Epoxy groups located on different fatty acids and on the same fatty acid, respectively. For abbreviation see Table 1.

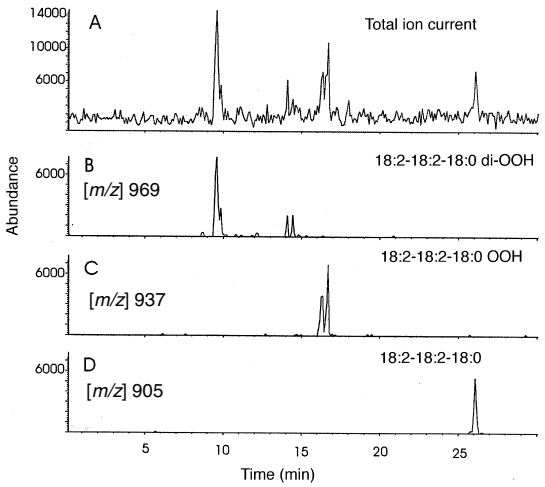


FIG. 1. Total ion chromatogram of the high molecular weight products from photosensitized oxidation of 1,2-dilinoleoyl-3-stearoyl-*sn*-glycerol as obtained by reverse-phase liquid chromatography/electrospray/mass spectroscopy (LC/ES/MS). Regioisomers of hydroperoxides are partly resolved as discussed in text. Mass values shown are for the sodium adducts $[M + Na]^+$ detected in the positive ion mode. Reverse-phase high-performance liquid chromatography (HPLC) column and instrumentation are described in the Materials and Methods section. Solvent gradient: 20–80% 2-propanol in methanol (0.85 mL/min) over 30 min.

with total ion plot. In this study, *cis, trans* or *trans,trans* linoleic 9- or 13-hydroperoxides were not resolved but yielded only one peak for oxidized 1,2-dipalmitoyl-*sn*-glycerol linoleate. Neff *et al*. (23) were able to separate and identify eight different regioisomers of monohydroperoxides in autoxidized trilinoleoylglycerol using analytical normal-phase HPLC. They showed also that bis- and trishydroperoxides were formed from further oxidized monohydroperoxides. Our system corresponds to prepative reverse-phase HPLC used by Neff *et al*. (23) for separation of mono-, bis-, and trishydroperoxides from unoxidized trilinoleoyl glycerol. However, regioisomers of monohydroperoxides were partly resolved, which can been seen in Figure 1. A single-ion plot of the monohydroperoxide of 1,2-dilinoleoyl-3-stearoyl (*m/z* 937) shows a double peak which is due to the *sn*-position of a hydroperoxy group in a glycerol molecule. We also oxidized individually regioisomers of 1,3-distearoyl-2-oleoyl and 1,2-distearoyl-3-oleoyl-*sn*-glycerols. Their hydroperoxy derivatives were first analyzed separately and then together, showing that regioisomers of hydroperoxides were partly resolved by LC/MS and HPLC with a light-scattering detector. The triacylglycerols with the hydroperoxy function in the *sn*-2-position were eluted ahead of their regioisomers, as well as ahead of the corresponding core aldehyde derivatives already discussed. Positional isomers of hydroperoxy triacylglycerols have been previously identified in autoxidized trilinolenoylglycerol preparations and in synthetic triacylglycerols containing linoleate (12,13) and linolenate (12).

The reduction of the mono- and dihydroperoxy triacylglycerols with sodium borohydride or with TPP yielded the corresponding hydroxides as single products, as indicated by reverse-phase LC/ES/MS and cochromatography with potential by-products. Their regioisomers were also partly separated as their hydroperoxy compounds. Monohydroperoxides showed approximately 3–5 times and dihydroperoxides about 10–20 times higher relative response as the $[M + NH_4]$ ions compared to the $[M + 1]^+$ ions of the corresponding unsaturated triacylglycerols on LC/ES/MS using neutral lipid solvents. The hydroperoxides also showed higher response than the corresponding parent compounds on reverse-phase HPLC with a light-scattering detector.

Epoxy triacylglycerol derivatives were prepared by a new method of epoxidation, which has been reported to yield quantitative transformation (26). The epoxidation of the unsaturated triacylglycerols yielded both mono- and diepoxides as the reaction products. The compounds were resolved from the parent triacylglycerols and the hydroperoxy and hydroxy derivatives by normal-phase TLC or reverse-phase HPLC. Regioisomers of monoepoxides were not separated by reverse-phase HPLC, although a slight difference was obtained in elution factor as they were estimated separately with a series of saturated triacylglycerols. However, triacylglycerols with two epoxy groups located in one linoleate eluted later than two epoxy groups located on two oleates in reverse-phase HPLC.

Determination of HPLC retention times. The relative retention times of the oxotriacylglycerols were determined by reverse-phase HPLC under optimal conditions of separation using a light-scattering detector, which gave nearly comparable intensities for equal masses of the solutes. The triacylglycerols of known structure were oxidized separately to give unambiguous products. The retention times were obtained by injecting (individually or in small sets) the oxotriacylglycerols together with the homologous series of the saturated triacylglycerols. A linear gradient of isopropanol/methanol 20–80% was found to be a good mobile phase for the resolution of the native and modified triacylglycerols, including the core aldehydes and their DNPH derivatives. Under these conditions, coefficients of variance of retention time ($n = 6$) ranged from 0.17 (triheptadecanoylglycerol) to 0.35% (trioctanoylglycerol) during elution times ranging from 6.12 to 33.73 min. The other homologous triacylglycerols showed variance of retention time within these extremes. Figure 2 shows the elution profile obtained for the homologous saturated triacylglycerols coinjected with the free core aldehydes [1,2-distearoyl-2-(9-oxo)nonanoyl-*sn*-glycerol (A) and 1,2-distearoyl-3-(9-oxo)nonanoyl-*sn*-glycerol (B)] and their DNPH derivatives (C and D, respectively). There is a clear indication of the separation of the positional isomers, with the 2-isomer emerging ahead of the 1(3)-isomer. On reverse-phase HPLC, the dialdehyde-containing triacylglycerols were eluted earlier than the monoaldehyde derivatives, as already established for other positional isomers of acylglycerols (17). Likewise, the dihydroperoxy triacylglycerols were eluted earlier than the monohydroperoxy derivatives, and the dihydroxytriacylglycerols earlier than the monohydroxy derivatives, and the diepoxytriacylglycerols earlier than the monoepoxytriacylglycerols. The hydroperoxide group was observed to have only slightly more polar effect in reverse-phase HPLC. A part of the 1,2-distearoyl-3-linoleoyl-*sn*-glycerol hydroperoxide was reduced to the corresponding hydroxytriacylglycerol, and they were analyzed together. The hydroperoxides were eluted just ahead of the corresponding hydroxytriacylglycerols.

Estimation of elution factors. The incremental elution factors of the oxotriacylglycerols were obtained by plotting the retention times, recorded using a linear gradient of 20–80% isopropanol/methanol, against the theoretical carbon number (TCN). TCN (2) was obtained from the equivalent carbon number (ECN) by adding a correction factor (F_i):

$$\text{TCN} = \text{ECN} + \Sigma\,(F_i) \qquad [1]$$

The ECN (2) was obtained by subtracting two carbon numbers (CN) for each double bond (n) present in the triacylglycerol molecule:

$$\text{ECN} = \text{CN} - 2\,n \qquad [2]$$

The curve-fitting procedure was adopted from Myher *et al*. (6). Saturated fatty acids were assumed to have a correction factor value of 0. A polynominal equation of fifth to seventh order was used to fit the TCN values of unsaturated triacylglycerols and oxotriacylglycerols to the elution times of the saturated triacylglycerols. A comparison of the TCN values

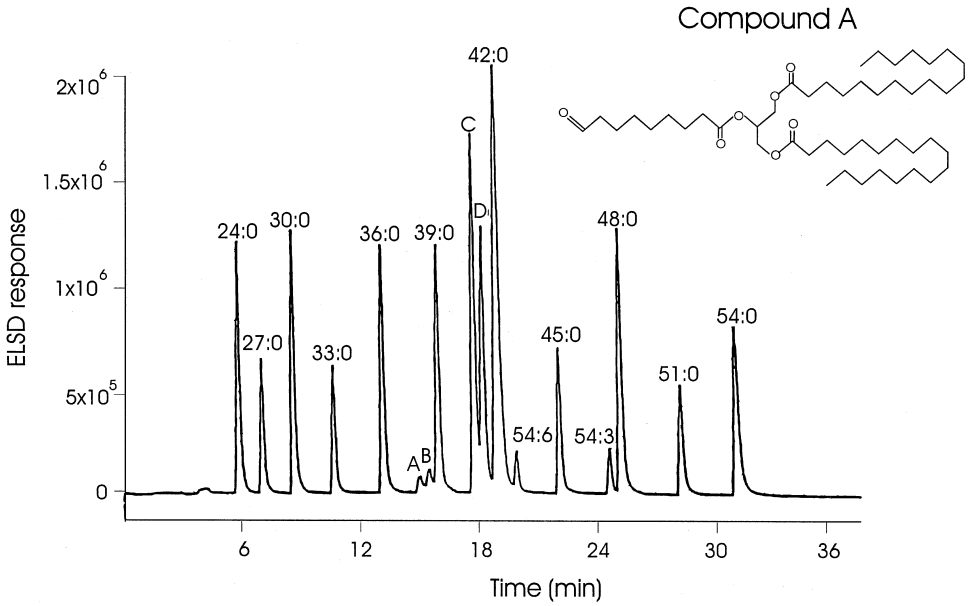


FIG. 2. Reverse-phase HPLC of homologous saturated triacylglycerols coinjected with the free core aldehydes and their 2,4-dinitrophenylhydrazine (DNPH) derivatives as detected by a light-scattering detector. Core aldehydes are identified as follows: A, 1,2-distearoyl-2-[9-oxo]nonanoyl-*sn*-glycerol; B, 1,2-distearoyl-3-[9-oxo]nonanoyl-*sn*-glycerol; C and D are DNPH derivatives of A and B, respectively. Triacylglycerol homologs are identified by their total acyl carbon:double-bond numbers. Abbreviation and reverse-phase HPLC conditions are given in Figure 1. ELSD, evaporative light-scattering detector.

calculated for the unsaturated and oxotriacylglycerols allowed one to estimate the effect of a double bond and a functional group upon the elution time. The calculated incremental elution factors are given in Table 3. As a result, a reference curve was obtained for the homologous series of saturated triacylglycerols and the oxotriacylglycerol standards vs. the retention time (Fig. 3). The various functional groups added to the triacylglycerol molecule, however, did not constitute totally independent contributions to the retention time, and therefore the calculated incremental elution factors differ slightly from the simple sums of the individual contributions. The functional groups apparently interacted with neighboring groups (30), and this interaction altered their polarity. Thus, for triacylglycerols containing two hydroperoxy groups, the elution factors showed a 7–9% reduction from the total sum of the elution factors estimated for monohydroperoxides, depending on the exact location of the substituents. Likewise, triacylglycerol dialdehydes yielded 9–16% lower elution factors than the sum of the individual elution factors of a monoaldehyde. That differences in elution factors were seen for 18:1*cis*9 and 20:1*cis*11 fatty acids was in agreement with earlier work (3,6). The elution factor observed for linoleic acid varied from 0.19 to 0.31, depending on the number of linoleic acids in a triacylglycerol molecule.

TABLE 3
Estimated Elution Factors for Functional Groups and Fatty Acids of Triacylglycerols

Fatty acid	One fatty acid	Two fatty acids[a]	Three fatty acids[a]
18:1	–0.51 ± 0.05	–0.74 ± 0.05	–0.55 ± 0.05
18:2	0.22 ± 0.05	0.38 ± 0.05	0.92 ± 0.05
18:3	0.77 ± 0.05	1.82 ± 0.05	
20:1	–0.64 ± 0.05		

Functional group	*sn*-1/3-position	*sn*-2 position	Two groups in *sn*-1/3- and *sn*-2-positions
–OOH (18:1)	–8.87 ± 0.1	–8.97 ± 0.1	–16.30 ± 0.1
–OOH (18:2)	–8.40 ± 0.1	–8.56 ± 0.1	–15.57 ± 0.1
–OH (18:1)	–8.46 ± 0.1	–8.71 ± 0.1	–15.30 ± 0.1
–OH (18:2)	–8.17 ± 0.1	–8.36 ± 0.1	–14.42 ± 0.1
–epoxy (18:1)	–4.55 ± 0.1	–4.62 ± 0.1	–8.73 ± 0.1
–epoxy (18:2)			–7.20 ± 0.1
–CHO	–6.51 ± 0.1	–7.04 ± 0.1	–11.79 ± 0.1
–CHO DNPH	–3.66 ± 0.1	–4.11 ± 0.1	–6.44 ± 0.1

[a]Total effect of double bonds or functional groups.

When a functional group was attached to a double bond, the polarity of the molecule was amplified, decreasing the retention time and TCN of a triacylglycerol molecule in reverse-phase HPLC, resembling the effect of bromine addition to a fatty acid moiety (3). Hydroperoxy groups on an oleic acid residue enhanced the polarity (eluted earlier) of a triacylglycerol by 0.4 carbons more than a hydroperoxy group on a linoleic acid residue in a similar triacylglycerol molecule (Fig. 4). The retention times did not seem to be affected by the positional location of the functional group on the fatty acid moiety. A hydroxyl group reduced the retention time of a hydroxytriacylglycerol by 8.2–8.7 carbons in our reverse-phase system. In another solvent system (11), the reduction in retention time had been estimated to be six carbons. The relative retention time and therefore the incremental elution factor are clearly affected by the solvent system. The elution factor of an epoxy group was about 50% of hy-

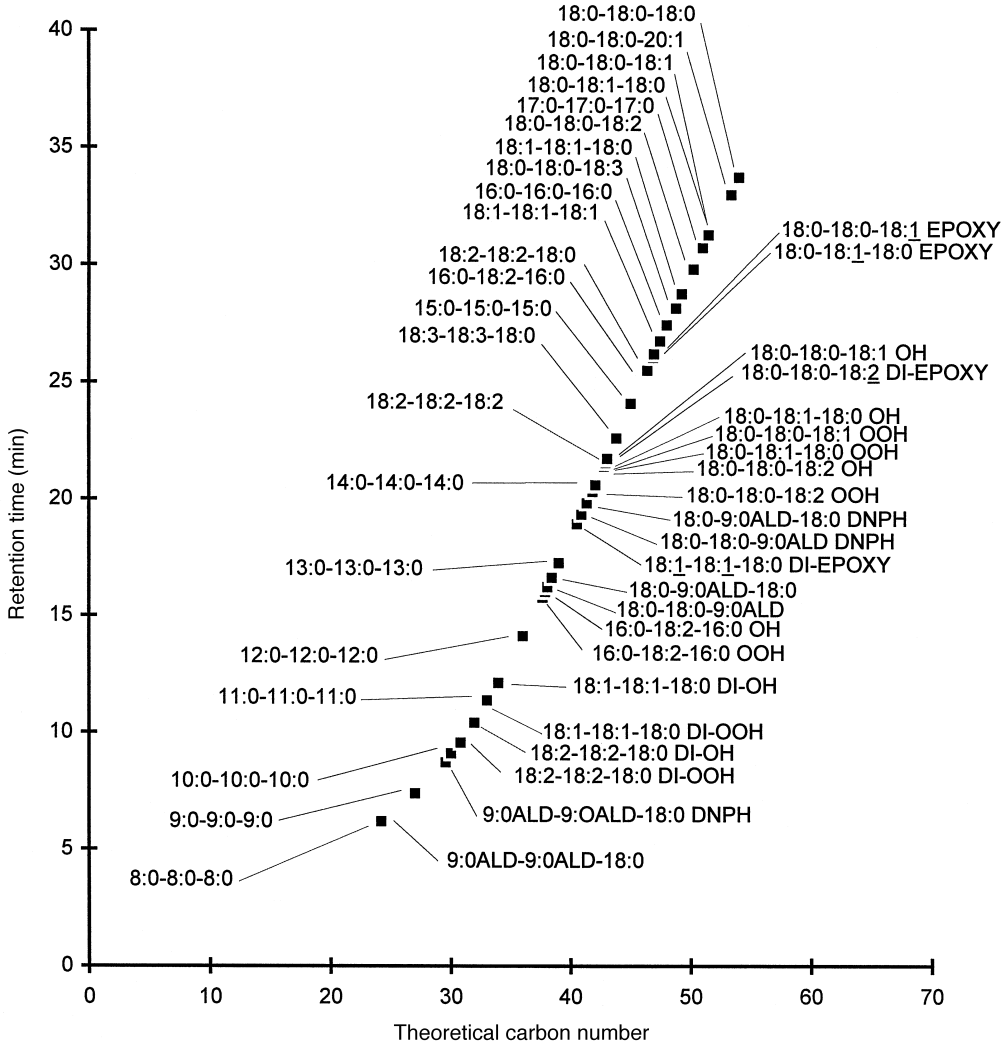


FIG. 3. A plot of theoretical carbon numbers (TCN) vs. retention times of reference compounds along with a series of saturated monoacid triacylglycerols. TCN and correction factors were calculated for oxotriacylglycerols and unsaturated triacylglycerols using saturated triacylglycerols as a reference curve. Unsaturated and oxotriacylglycerols were co-injected individually or in small sets with the series of the saturated triacylglycerols. Abbreviation: ald, aldehyde. For other abbreviation see Figure 2. Position of an epoxy group is marked as an underlined double bond.

droxyl group, which is in accordance with earlier results (31). The accuracy of the estimates of the incremental elution factors could be further improved by preparing reference standards containing two or more homologous or heterologous functional groups per triacylglycerol molecule. Nevertheless, the knowledge of even a limited set of elution factors allows the calculation of the relative retention times of unknowns with considerable accuracy, which helps to choose among likely structures represented by the same molecular weight during reverse-phase LC/ES/MS in oxidized vegetable oils as shown elsewhere (32).

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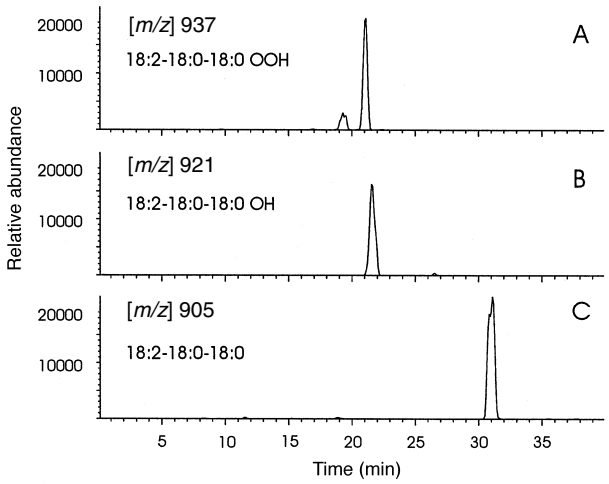

FIG. 4. Single-ion plots of (C) 1,2-distearoyl-3-linoleoyl-*sn*-glycerol and its hydroperoxy (A) and hydroxy (B) derivatives, demonstrating that the oxide elutes slightly ahead of the alcohol during reverse-phase HPLC. Mass values shown are for the ammonia adducts $[M + NH_4]^+$ detected by positive-ion LC/ESI/MS. Reverse-phase HPLC conditions are described in Figure 1. LC/ES/MS conditions are given in the Materials and Methods section.

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Evaluating Acid and Base Catalysts in the Methylation of Milk and Rumen Fatty Acids with Special Emphasis on Conjugated Dienes and Total *trans* Fatty Acids

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ABSTRACT: Milk analysis is receiving increased attention. Milk contains conjugated octadecadienoic acids (18:2) purported to be anticarcinogenic, low levels of essential fatty acids, and *trans* fatty acids that increase when essential fatty acids are increased in dairy rations. Milk and rumen fatty acid methyl esters (FAME) were prepared using several acid- (HCl, BF_3, acetyl chloride, H_2SO_4) or base-catalysts ($NaOCH_3$, tetramethylguanidine, diazomethane), or combinations thereof. All acid-catalyzed procedures resulted in decreased *cis/trans* (Δ9*c*,11*t*-18:2) and increased *trans/trans* (Δ9*t*,11*t*-18:2) conjugated dienes and the production of allylic methoxy artifacts. The methoxy artifacts were identified by gas–liquid chromatography (GLC)-mass spectroscopy. The base-catalyzed procedures gave no isomerization of conjugated dienes and no methoxy artifacts, but they did not transesterify *N*-acyl lipids such as sphingomyelin, and $NaOCH_3$ did not methylate free fatty acids. In addition, reaction with tetramethylguanidine coextracted material with hexane that interfered with the determination of the short-chain FAME by GLC. Acid-catalyzed methylation resulted in the loss of about 12% total conjugated dienes, 42% recovery of the Δ9*c*,11*t*-18:2 isomer, a fourfold increase in Δ9*t*,11*t*-18:2, and the formation of methoxy artifacts, compared with the base-catalyzed reactions. Total milk FAME showed significant infrared (IR) absorption due to conjugated dienes at 985 and 948 cm^{-1}. The IR determination of total *trans* content of milk FAME was not fully satisfactory because the 966 cm^{-1} *trans* band overlapped with the conjugated diene bands. IR accuracy was limited by the fact that the absorptivity of methyl elaidate, used as calibration standard, was different from those of the other minor *trans* fatty acids (e.g., dienes) found in milk. In addition, acid-catalyzed reactions produced interfering material that absorbed extensively in the *trans* IR region. No single method or combination of methods could adequately prepare FAME from all lipid classes in milk or rumen lipids, and not affect the conjugated dienes. The best compromise for milk fatty acids was obtained with $NaOCH_3$ followed by HCl or BF_3, or diazomethane followed by $NaOCH_3$, being aware that sphingomyelins are ignored. For rumen samples, the best method was diazomethane followed by $NaOCH_3$.

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Interest in milk fatty acid composition was renewed because of ongoing studies to increase the essential fatty acid content (1,2), to determine accurately the *trans* content (3–8), and lately, to quantitate the conjugated octadecadienes (9–15) which have been associated with inhibition of carcinogenesis and tumorigenesis (16–18). Milk and rumen fatty acid compositions are complex (1,19–21). Not only is there a large range in chain length from C_4 to C_{26}, including branch-chain fatty acids, but milk also contains many positional and geometric isomers of mono-, di-, and tri-unsaturated fatty acids, and many of these fatty acids are present in very low concentrations. In 1991, Jensen *et al.* (19) estimated 400 fatty acids to be present in bovine milk.

No single method is presently able to resolve all 400 fatty acids. The availability of long polar capillary columns (50 to 100 m) for gas–liquid chromatography (GLC) has improved the resolution of many positional and geometric fatty acid isomers (3–8,10–12,15,22). Prior separations with argentation chromatography still show several regions with overlapping peaks particularly in the mono- and diunsaturated fatty acid region (3–8,22–25). The development of a routine method for the analysis of total milk and rumen fatty acids, without extensive secondary fractionations, would be desirable.

Difficulty may occur in quantitatively preparing esters from such a great variety of fatty acids and lipid classes that are present in milk and rumen lipids. Sodium methoxide ($NaOCH_3$)-catalyzed methylations have been used (3,9), but free fatty acids and *N*-acyl lipids (i.e., sphingolipids and glycosphingolipids) are not methylated under these conditions (26). Acid-catalyzed methylations convert all known lipid classes; however, reports have indicated that the conjugated dienes are isomerized (12,27–29). The very short-chain fatty acid methyl esters (FAME) are difficult to quantitate because the methyl esters are volatile, water-soluble, and require correction factors (30,31). Some of the difficulties with the very

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Abbreviations: ATR, attenuated total reflection; FTIR, Fourier transform infrared; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; IR, infrared; MS, mass spectrometry; TLC, thin-layer chromatography.

short-chain fatty acids were avoided by preparing H_2SO_4-catalyzed isopropyl esters for milk analyses (4,6,8,24,32).

In the present study, several acid- and base-catalyzed methylation methods were evaluated for the analyses of milk and rumen lipids, including a combination of methods. The methods included the following catalysts in anhydrous condition: HCl/methanol (33), BF_3/methanol (34), acetyl chloride/methanol (35), $NaOCH_3$/methanol (36), tetramethylguanidine/methanol (13,27,37), H_2SO_4/isopropanol (4,6,24,32), and diazomethane in diethyl ether/methanol (26). Several criteria were considered in evaluating methods for the analysis of milk and rumen lipids: (i) completion of methylation of free fatty acids and *O*- and *N*-esters as judged by thin-layer chromatography (TLC); (ii) coextraction of artifacts with hexane that may interfere with the direct analysis of the hexane extract by GLC; (iii) extent of isomerization of conjugated dienes and methoxy artifact formation as judged by GLC; and (iv) comparison of total *trans* content determined by GLC and infrared (IR) spectroscopy. A chromatographic separation is presented using a 100-m capillary column that resulted in the separation of about 180 peaks from milk lipids.

MATERIALS AND METHODS

Materials. Milk and rumen samples were obtained from cows used in two separate experiments to test the effect of ionophores on milk and rumen fatty acid composition. Other reports have detailed the effect of ionophores on rumen and milk lipids (38–40). Methyl ester (Nu-Chek-Prep, Inc., Elysian, MN) and phospholipid (Matreya Inc., Pleasant Gap, PA) standards were obtained commercially.

Extraction of lipids. Total rumen lipids were extracted with chloroform/methanol (2:1, vol/vol) as described previously (38). Total milk lipids were extracted with a modified Folch procedure described by Jensen (41). The total lipids were taken up in 10 mL of chloroform and stored at −70°C until analyzed.

Preparation of fatty acid esters. The total rumen and milk lipids were methylated in 15-mL culture tubes equipped with Teflon-lined screw caps, under N_2, and in anhydrous conditions. HCl/methanol (33), acetyl chloride/methanol (35), and H_2SO_4/isopropanol (6,32) were heated for 1 h at 80°C. $NaOCH_3$/methanol (36) and 14% BF_3/methanol (34) were heated for 10 min at 50°C. Tetramethylguanidine/methanol was heated for 2 min at 100°C (27,37). Diazomethane in diethyl ether/methanol was left for 10 min at room temperature (26). The excess diazomethane and solvents were then removed by a stream of nitrogen and the residue dissolved in 2 mL hexane and analyzed directly by GLC. To all other reactions, 5% water was added to the alcohol solution, and the esters were extracted with 2 mL of hexane and analyzed directly by GLC. To avoid any loss of short-chain esters, the hexane layer was not reduced in volume. Base-catalyzed reactions were neutralized with aqueous HCl prior to hexane extraction. To ensure completion of reaction, the total lipids were extracted using the Bligh and Dyer mixture (42). The extent of neutral lipid esterification was checked by TLC using the developing solvent hexane/diethyl ether/acetic acid (85:15:1, by vol). The polar lipids were checked by TLC using the developing solvent chloroform/methanol/water (65:25:4, by vol). The FAME were extracted with hexane and analyzed directly by GLC to determine the extent of isomerization of conjugated dienes.

Several combinations of methylations were also tested with standard neutral- and phospholipids mixtures: $NaOCH_3$/methanol for 10 min at 50°C followed by an excess of HCl/methanol for 10 min at 80°C; $NaOCH_3$/methanol for 10 min at 50°C followed by an excess of BF_3/methanol (43) for 10 min at 50°C; and diazomethane for 10 min at room temperature followed by evaporation to dryness in a stream of N_2, and reaction with $NaOCH_3$/methanol for 10 min at 50°C. The extent of methylation of the total lipid extract was checked by TLC, while the extent of isomerization of the conjugated dienes was checked by GLC.

Analyses of esters by GLC. FAME were analyzed by GLC (model 5890; Hewlett-Packard, Palo Alto, CA) using a SP-2560 fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness; Supelco Inc., Bellefonte, PA), H_2 as the carrier gas, a 1:15 split mode, and a flame-ionization detector. For comparison, FAME were also analyzed using a CP-Sil 88 WCOT fused silica column (100 m × 0.25 mm i.d. × 0.2 μm film thickness; Chrompack, Middleburg, The Netherlands). Both columns were operated at 70°C for 4 min, then temperature-programmed at 13°C/min to 175°C, held there for 27 min, programmed at 4°C/min to 215°C, and finally held there for 31 min; total run was 80 min. Starting at 70°C permitted the resolution of the short-chain FAME. Maintaining the temperature at 215°C for a longer period of time allowed the emergence of the very long-chain FAME. The temperature program for the isopropyl esters was similar to FAME except that it was started at 90°C, taken to 195°C, and held at 215°C for 36 min.

Fractionation of FAME by high-performance liquid chromatography (HPLC). Total milk and rumen FAME were separated on a reverse-phase Supelcosil™ LC-8DB column (25 cm × 4.6 mm i.d.; 5-μm particle size; Supelco Inc.) using an HPLC (model 1090; Hewlett-Packard) equipped with an autosampler. The system was operated at room temperature with a flow rate of 1.5 mL/min. A portion of the eluate was diverted (three-way valve #02-0124; Alltech Associates Inc., Deerfield, IL) to an evaporative light-scattering detector (model MKIII; Varex Alltech Associates Inc.) operated at 75°C and 2.5 L/min of N_2, and the remainder was collected using a fraction collector (Gilson model 203; Mandel Scientific Co. Ltd., Guelph, Ontario, Canada). Approximately 1 mg of total lipids in 25 μL hexane was applied onto the column. Fractions were eluted isocratically with 87.5% methanol and 12.5% water and collected at 1-min intervals from 3 to 35 min. After each run, the column was regenerated with dichloroethane.

Analyses of FAME by GLC–FTIR (Fourier transform infrared) spectroscopy and GLC–MS (mass spectrometry). The same GLC conditions as described above were used to ana-

lyze the FAME by GLC–FTIR using a Hewlett-Packard system (models 5890 and 5965B) as described previously (38). In addition, the FAME were analyzed by a GLC–MS system (model 4500; Finnigan, San Jose, CA) using the same GLC conditions. Mass spectra were acquired in 1-s increments ranging in mass from 30 to 500.

Measurement of trans *absorption of FAME by attenuated total reflection (ATR)–FTIR spectroscopy.* An FTIR spectrometer (model FTS-60A; Bio-Rad, Digilab Division, Cambridge, MA) was used equipped with a Graseby Specac (Fairfield, CT) Golden Gate Single Reflection Diamond ATR cell that required about 1 µL test sample. This accessory contained a 2×2 mm diamond with an active sampling area of 0.6 mm in diameter. It was prealigned in the factory, and preattached to its baseplate that attached to the floor of the FTIR spectrometer sample compartment. Mounting holes and pegs in the baseplate ensured its proper positioning and alignment.

Neat (without solvent) FAME test aliquots (1 µL) were placed without weighing on the horizontal (face-up) diamond sampling surface of the cell. For quantitative work, the test aliquots had to cover all of the horizontal surface of the diamond. Two hundred and sixty-four scans were collected at 4 cm^{-1} resolution. After a measurement was made, the surface of the diamond was cleaned by wiping it with low-lint paper.

ATR quantitation was based on the measurement of the integrated area between the limits of 990 and 945 cm^{-1}, which included all C-H out-of-plane deformation absorptions of isolated *trans* double bonds, and *trans* contained in methylene and nonmethylene-interrupted 18:2 isomers at 966 cm^{-1}. The contribution of overlapping conjugated *cis,trans* and *trans,trans* diene bands at 985 and 948 cm^{-1} (44) was subtracted digitally. A calibration plot of "area" vs. "percentage" *trans* FAME was generated for reference mixtures in the range of 0.81–14.4% methyl elaidate in methyl oleate which was used as the infrared (IR) reference material. The regression line parameters for the *trans* standards were 0.001194 (intercept) and 0.01777 (slope), with a correlation coefficient of 0.9996. A commercial FAME reference mixture was used which consisted of 16:0 (6%), 18:0 (3%), 18:1n-9 (35%), 18:2n-6 (50%), 18:3n-3 (3%), and 20:4n-6 (3%) (#RM-1; Matreya Inc., Pleasant Gap, PA); all double bonds were *cis.* Absorption spectra were obtained by "ratioing" the test sample single-beam spectra against that of the reference mixture. For test samples, the percentage of total *trans* was calculated from the absorption area between the limits of 990 and 945 cm^{-1}, and the linear regression equation that described the calibration plot. The lower limit of quantitation was about 1%. Using cells with 1.5 mL and 50 µL capacity, the ATR method was successfully used to determine the *trans* levels in hydrogenated oils and commercial food products (45,46), and this method was recently adopted as Recommended Practice (47).

Statistics. Milk and rumen FAME were analyzed by the analysis of variance.

RESULTS AND DISCUSSION

Analysis of milk FAME by GLC. A typical GLC chromatogram of total milk FAME using a 100-m polar fused silica capillary column (SP-2560; Supelco Inc.) is shown in Fig-

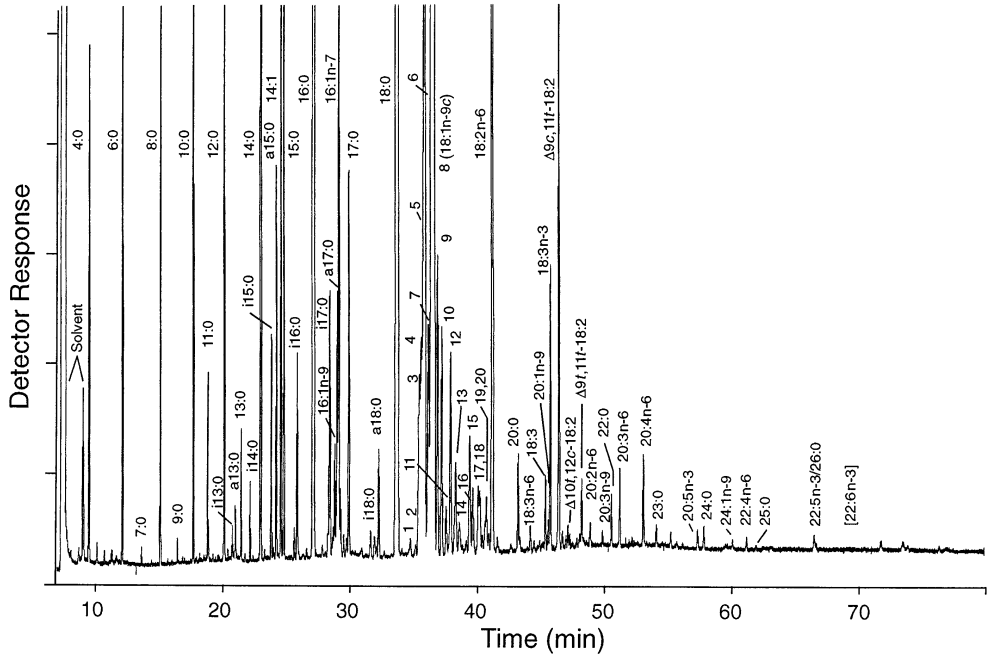

FIG. 1. A typical gas–liquid chromatogram of total bovine milk fatty acid methyl ester prepared by $NaOCH_3$/methanol (10 min at 50°C) followed by HCl/methanol (10 min at 80°C), and separated on a 100-m fused silica capillary column (SP-2560; Supelco Inc., Bellefonte, PA). i, iso; a, anteiso; numbers 1–20 are an arbitrary consecutive numbering of all the peaks in the region between 18:0 and 18:2n-6 using this column. [22:6n-3] is not present in these milk samples but the position at which it should emerge in the chromatogram is indicated.

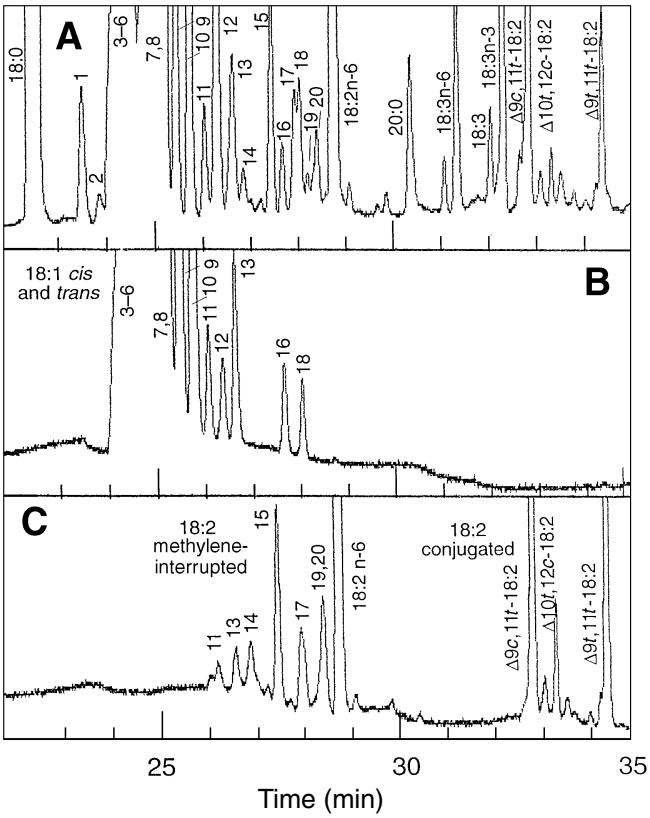

FIG. 2. The methyl stearate (18:0) to conjugated octadecadiene region of the gas–liquid chromatogram of total bovine milk fatty acid methyl esters (A), and methyl octadecenoates [B; emerged at 8 min from high-performance liquid chromatography (HPLC) column] and methyl octadecadienoates (C; emerged at 11 min from HPLC column) isolated from total milk fatty acid methyl esters by HPLC. Gas–liquid chromatography (GLC) analysis was on the same type of column as Figure 1. Numbers 1–20 refer to the same numbers as in Figure 1 between 18:0 and 18:2n-6.

ure 1. Using a temperature program from 70 to 215°C permitted a separation from the short-chain (4:0) to the very long-chain saturated (26:0) and polyunsaturated (20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-3) FAME found in milk. A total of about 180 peaks were observed. Many of the peaks were identified by comparison to known FAME standards and confirmed by GLC–MS and GLC–FTIR. The 100-m CP-Sil 88 WCOT fused silica column gave similar results to the SP-2560 column with a few minor exceptions. There appeared to be a better resolution of *trans*-16:1 and iso-17:0, anteiso-17:0 and 16:1n-7, and 20:1n-9 and 18:3n-3 with the CP-Sil 88 column, while the resolution of 22:1n-9 and 20:3n-3, and 20:5n-3 and 24:0 was better with the SP-2560 column. Resolution of critical pairs was sometimes improved by changing the column temperature, as shown previously (48). However, that process was limited, because a compromise was needed to deal with the wide range of fatty acids present in milk and rumen lipids.

The major unresolved peaks with either column occurred between 18:0 and 18:2n-6 and included several positional isomers of *cis*- and *trans*-18:1, and the methylene-interrupted and noninterrupted 18:2 isomers, many of them overlapping. The 18:1–18:2 region was resolved by HPLC into mono- and diunsaturated FAME (Fig. 2). With the use of GLC–FTIR, the identity of the major *cis* and *trans* absorption peaks was determined, but not the minor peaks (i.e., peaks 1,2,11,13–20). Based on the HPLC separation and GLC–FTIR results, the peaks in 18:1–18:2 region were identified as *trans*-18:1 (peaks 3–7, and part of peak 10), *cis*-18:1 (peaks 8–13,16,18), and 18:2-isomers (peaks 11,13–15,17,19,20). No attempt was made to further separate *cis*- and *trans*-18:1 or identify each of the isomers, since this was adequately described by others in recent publications on milk and milk fat lipids (4–8,22–25).

Analysis of conjugated dienes using either base- or acid-catalyzed methylations. The composition of the conjugated dienes was greatly affected by the catalyst used in the methylation procedure. Base-catalyzed methylation of milk using $NaOCH_3$ showed Δ9*c*,11*t*-18:2 as the major conjugated diene, with smaller amounts of eight other isomers including Δ9*t*,11*c*-18:2, Δ10*t*,12*c*-18:2, Δ9*c*,11*c*-18:2, and Δ9*t*,11*t*-18:2 (Fig. 3). On the other hand, all acid-catalyzed methylation procedures resulted in decreased Δ9*c*,11*t*-18:2 and increased Δ9*t*,11*t*-18:2, and the formation of additional peaks between 20:2n-6 and 20:3n-9 (Fig. 3). The peaks formed during acid-catalyzed methylation were identified as positional allylic methoxy isomers of 18:1 by GLC–MS. All the methoxy artifacts had molecular ion at *m/z* 326. These allylic methoxy compounds could have been formed from hydroxy 18:1 present in milk (19). We also confirmed that these allylic methoxy derivatives were formed from the conjugated dienes by heating the standard methyl esters of Δ9*c*,11*t*-18:2 and Δ9t,11t-18:2 with HCl/methanol for 1 h at 80°C. The GLC and GLC–MS of the resultant FAME showed the formation of the corresponding methoxy derivatives as major product (data not shown). The formation of methoxy artifacts during acid-catalyzed methylations of conjugated dienes was first pointed out in a letter to the editor of *Lipids* as early as 1972 (29), but there was no follow-up manuscript, and its findings appeared to be largely ignored by many investigators. Similar allylic methoxy derivatives were reported recently following acid-catalyzed methylation of allylic hydroxy oleates (49). The GLC–MS results, isomer identification, and possible mechanism of formation of these allylic methoxy derivatives formed during acid-catalyzed methylation of milk lipids will be presented in a separate publication.

Acid-catalyzed methylation has been used extensively for the analysis of conjugated dienes in milk and dairy products. The catalysts used by several groups included HCl (16,17,28), acetyl chloride (20,27,35,50), BF_3 (5,10–12,15,20,22,23,27, 32,43,48,50), and H_2SO_4 (4,6,8,24,32). Some authors recognized the isomerization of the conjugated dienes during acid-catalyzed methanolysis and recommended that the reaction of BF_3/methanol (12,15) or HCl/methanol (28) be carried out at room temperature to reduce this isomerization. Our TLC results showed that methylation was not complete with either BF_3 or HCl as a catalyst under these mild conditions (data not shown). GLC analyses showed evidence of isomerization and artifact (methoxy) for-

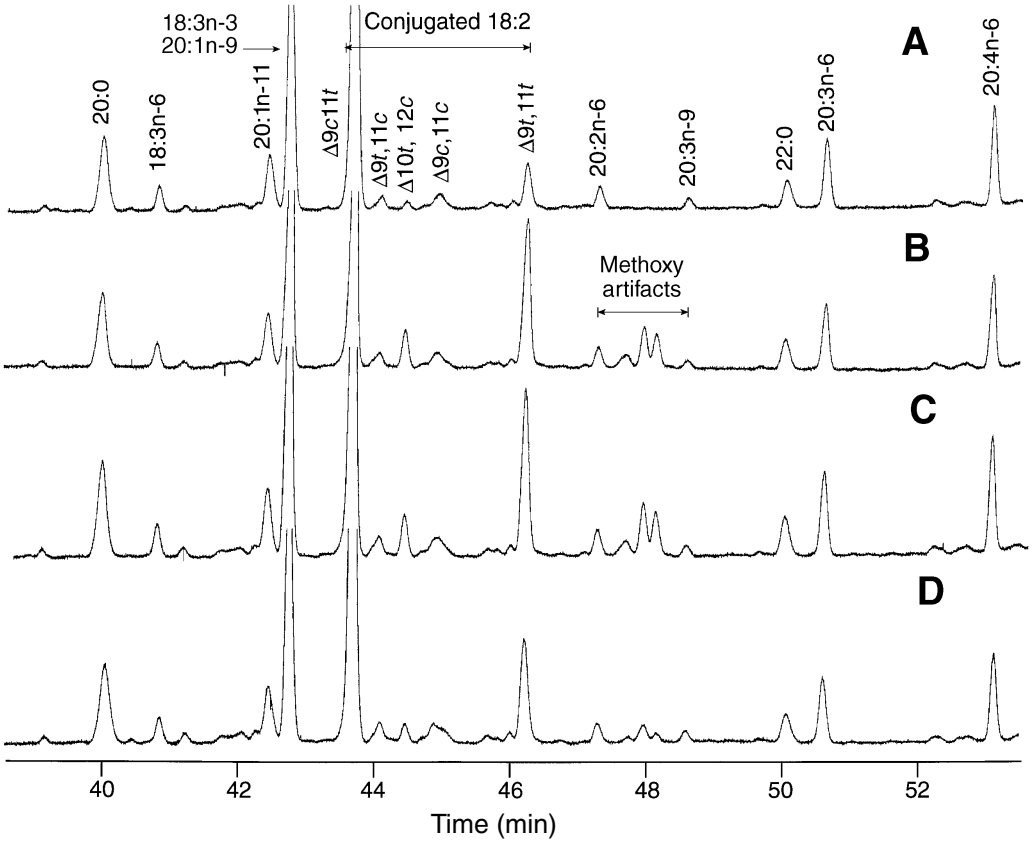

FIG. 3. The methyl eicosanoate (20:0) to methyl arachidonate (20:4n-6) region of the GLC of total bovine fatty acid methyl esters prepared by using the following catalysts in anhydrous methanol: (A) $NaOCH_3$, (B) HCl, (C) acetyl chloride, or (D) BF_3. Analysis was on the same type of GLC column as Figure 1. See Figure 2 for abbreviation.

mation. In one of the milk samples, the ratio of Δ9*c*,11*t*-18:2 to Δ9*t*,11*t*-18:2 was 3.6 with $NaOCH_3$ and 2.7 with either HCl or BF_3 at room temperature. Therefore, not even mild acid-catalyzed methylation at room temperature is appropriate for the analysis of conjugated dienes. Furthermore, it should be pointed out that in two publications additional peaks were observed in the GLC chromatogram of conjugated dienes after acid-catalyzed methylation (12,27), but the peaks were not identified.

In several recent publications, Wolff's group recommended the preparation of isopropyl esters using H_2SO_4 as catalyst (4,6,8,24,32). This method was proposed to overcome the need for the use of correction factors for the short-chain FAME (24,30,32) and to improve the resolution of 4:0 from the solvent front (4). However, this method did not address the issue of isomerization of conjugated dienes. Reevaluation of H_2SO_4-isopropanol methylation reaction showed extensive isomerization of conjugated dienes and artifact formation, just as for HCl/methanol. In one publication (32) isopropyl esters were prepared by acid (H_2SO_4) and base (Na) catalysts, but no comparison was made of the distribution of 18:2 *cis,trans* conjugated dienes. However, their results showed a higher content of total conjugated dienes (18:2 *c,t*) with base- than with acid-catalyzed methylation (Table V, Ref. 32), which was similar to our finding (Table 1).

Recently, Shantha *et al.* (27) proposed the use of tetramethylguanidine, a base-catalyzed reagent developed by Schuchardt and Lopes (37), for the analysis of conjugated dienes. It was reported that tetramethylguanidine caused little isomerization of the *cis/trans* to the *trans/trans* conjugated dienes (27) and that free fatty acids were methylated (27,37). At first glance, this method appeared to be an ideal methylation reagent for total milk lipids. However, we found that tetramethylguanidine gave incomplete methylation of phosphatidylcholine, and did not methylate sphingomyelin as judged by TLC. Furthermore, hexane coextracted unknown material after the reaction which interfered with the determination of 4:0, and 8:0 to 10:0, present in milk. Introducing a water wash was unsuccessful in removing the interfering broad peaks, but a significant reduction was observed in the content of 4:0 and 6:0 FAME after the extra wash. For these reasons, tetramethylguanidine alone was not considered suitable for the methylation of total milk lipids.

Evaluating a combination of catalysts for the methylation of total milk lipids. Total milk lipids contain about 98% triacylglycerols, 1% phospholipids (phosphatidylcholine, phosphatidylethanolamine and sphingomyelin in about equal proportions), 0.3% cholesterol, 0.3% diacylglycerols, and trace amounts of cholesteryl esters and free fatty acids (20). Methylation using only $NaOCH_3$/methanol gave no isomerization of conjugated dienes and artifact formation, but did not methylate free fatty acids and sphingomyelin. The AOCS

TABLE 1
Fatty Acid Composition (area %) of Milk Lipids from Cows Fed Control or Ionophore-Treated Diets (for 2 wk). Comparison of Transesterification Procedures

Fatty	Control		Ionophore-treated		
acids (FA)	$NaOCH_3$	HCl	$NaOCH_3$	HCl	SE[e]
4:0–13:0	10.9[f]	10.2	10.2	9.8	0.7
14:0–18:0	48.0	46.9	47.1	47.4	0.7
20:0–26:0	0.29[b]	0.38[a]	0.28[b]	0.37[a]	0.01
iso FA[g]	1.02	1.00	1.03	1.00	0.02
anteiso FA[h]	0.71	0.70	0.71	0.71	0.01
18:1 *trans*(*t*)	3.6[b]	3.6[b]	6.3[a]	6.0[a]	0.1
18:1 *cis*(*c*)	26.5[a]	26.8[a]	23.5[b]	23.0[b]	0.4
18:2 *c,t*[i]	0.25[d]	0.49[c]	0.65[a]	0.61[b]	0.01
18:2n-6	3.6[b]	3.6[b]	3.8[a]	3.8[a]	0.05
18:2 conjugated	0.74[c]	0.67[d]	1.38[a]	1.21[b]	0.02
Δ9*c*,11*t*-18:2	0.52[b]	0.23[d]	1.08[a]	0.44[c]	0.03
Δ9*t*,11*t*-18:2	0.07[d]	0.25[b]	0.12[c]	0.48[a]	0.01
Δ9*c*,11*t*/9*t*,11*t*[j]	7.2[b]	0.9[c]	9.1[a]	0.9[c]	0.2
Methoxy artifacts[k]	0[c]	0.21[b]	0[c]	0.41[a]	0.01
n-6 PUFA[l]	0.29[b]	0.32[a]	0.29[b]	0.33[a]	0.01
n-3 PUFA[m]	0.53[b]	0.59[a]	0.54[b]	0.63[a]	0.01

[a-d]Means within a row with different superscript letters are different ($P < 0.05$).
[e]SE, pooled standard error.
[f]Each value represents the mean of the milk from 10 cows/diet. The same milk fat extract was transesterified with either $NaOCH_3$/methanol or HCl/methanol.
[g]Sum of all iso fatty acids: 13:0, 14:0, 15:0, 16:0, 17:0, and 18:0.
[h]Sum of all anteiso fatty acids: 13:0, 15:0, 17:0, and 18:0.
[i]Sum of all 18:2 methylene-interrupted *c,c, c,t, t,c,* and *t,t* isomers.
[j]Ratio of Δ9*c*,11*t*-18:2 to Δ9*t*,11*t*-18:2.
[k]Sum of all allylic methoxy artifacts.
[l]Sum of all n-6 PUFA (polyunsaturated fatty acids): 20:2n-6, 20:3n-6, 20:4n-6, and 22:4n-6.
[m]Sum of all n-3 PUFA: 18:3n-3, 20:5n-3, and 22:5n-3.

TABLE 2
Comparison of *trans* Content (% of total fatty acid methyl esters) of Milk from Two Separate Studies in Which Cows Were Treated with Ionophores (for 2 or 3 wk[a]).

		Control[b]		2 Wk		3 Wk	
Expt.	Method	$NaOCH_3$	HCl	$NaOCH_3$	HCl	$NaOCH_3$	HCl
1	GLC[c]	4.9[d]	5	8.3	8.2		
	GLC – CD[e]	4.1	4.3	6.9	6.9		
	IR[f]	5.8[g]	4.6	8.9	6.2[h]		
2	GLC	5.2	5.3	13.8	14.8	13.5	14.2
	GLC – CD	4.4	4.4	12.8	13.3	12.4	12.9
	IR	5.1	3.9[h]	12.6	6.7[h]	12.7	7.0[h]

[a]As measured by gas–liquid chromatography (GLC) and attenuated total reflection infrared (IR) spectroscopy. The experimental conditions of these studies are published elsewhere (Ref. 40).
[b]The total fat extract of each milk sample was analyzed by both methods.
[c]The GLC method involved summing all *trans* monounsaturates (peaks 3–7, half of 10; Fig. 1), *cis/trans* 18:2 (peaks 14,15,17,19,20; Fig.1) and total conjugated dienes.
[d]Each GLC value represented the mean of the milk from 10 cows.
[e]Total *trans* determined by GLC ([c]) minus total conjugated dienes (CD).
[f]The IR values represented the total *trans* absorption from monoenes, and methylene- and nonmethylene-interrupted 18:2. Conjugated dienes were excluded, see text.
[g]Each IR value represented the mean of two determinations. Milk samples 1 to 5 and 6 to 10 were combined because of insufficient material.
[h]Acid-catalyzed methanolysis showed interferences in the *trans* IR region, see text.

method recommends a combination of $NaOCH_3$ and BF_3/methanol (43). We tried the combination of $NaOCH_3$ (10 min at 50°C) followed by either BF_3/methanol (10 min at 50°C), or HCl/methanol (10 min at 80°C). The results showed that both these methods methylated free fatty acids, and most of the sphingomyelin as judged by TLC (data not shown). The GLC results showed that the ratio of Δ9*c*,11*t*-18:2 to Δ9*t*,11*t*-18:2 was only slightly reduced, with the formation of trace amounts of methoxy derivatives. This combination of methods appears to be the best compromise for the methylation of total milk lipids. A saponification followed by diazomethane has been used successfully for the analysis of conjugated dienes (51), but this method also ignores the sphingomyelins which are not saponified.

Quantitative analysis of conjugated dienes and trans *FAME of milk.* A summary of the milk fatty acid composition determined by using either $NaOCH_3$/methanol or HCl/methanol is compared in Table 1. Milk samples were obtained from ongoing studies at Agriculture and Agri-Food Canada to test the effect of ionophores on milk fatty acid composition (39,40). The most noted difference between methods was on the complex mixture of conjugated diene isomers (Fig. 3). $NaOCH_3$/methanol gave ~12% higher values of total conjugated dienes compared with HCl/methanol (Table 1). Of the total conjugated dienes, ~74% consisted of the Δ9*c*,11*t* isomer using $NaOCH_3$/methanol, compared with only ~35% when HCl/methanol was used, while the Δ9*t*,11*t* isomer increased from ~9% with $NaOCH_3$ to ~39% with HCl. The ratio of Δ9*c*,11*t* to Δ9*t*,11*t* for the two methods decreased from ~8 to 0.9 (Table 1). Some of the conjugated dienes were converted to allylic methoxy derivatives during acid-catalyzed methylation. However, the sum of the conjugated dienes plus the methoxy derivatives in acid-catalyzed methanolysis gave ~18% higher values than those observed for the $NaOCH_3$/methanol method (Table 1). This suggests that some of the methoxy derivatives may have been derived from preexisting hydroxy fatty acids (49). The methoxy artifacts did not appear to be derived from either 18:1 (*cis* or *trans*) or 18:2n-6 in milk during these acid methylation conditions, since the levels of these fatty acids were not significantly different between the two methods; see Table 1. In addition to the changes in conjugated dienes, HCl/methanol also gave slightly higher values for the long-chain saturated (20:0–26:0) and polyunsaturated fatty acids (n-6 and n-3) compared with $NaOCH_3$/methanol (Table 1). This was not surprising, since the acid catalyst methylated all the lipid components, including sphingomyelin rich in long-chain saturates, and free fatty acids plus minor acidic lipids which also contain polyunsaturated fatty acids.

Based on the GLC analyses, the total *trans* content of milk samples was similar using either $NaOCH_3$ or HCl as catalyst (Table 2). The total *trans* content was obtained by adding the relative percentage of all *trans* monounsaturated fatty acids (peaks 3–7, and half of peak 10; Fig. 1), *cis/trans* 18:2 (peaks 14,15,17,19,20; Fig. 1), and total conjugated dienes in the milk chromatograms (Table 2). In the milk samples investigated, the *trans* content increased from about 5 to 8% (Experiment 1, Table 2), and from about 5 to 14% (Experiment 2,

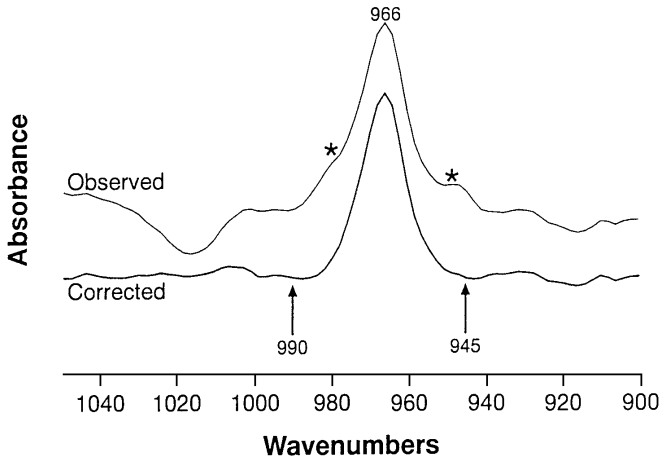

FIG. 4. Expanded infrared spectral range showing the out-of-plane deformation bands of isolated *trans* (966 cm^{-1}), and "*trans*-containing" conjugated dienes (985 and 948 cm^{-1}, denoted by asterisks) present in total bovine milk fatty acid methyl esters prepared by $NaOCH_3$/methanol (observed). The total *trans* area was integrated between the limits of 990 and 945 cm^{-1}, after subtracting the contribution of the conjugated dienes (corrected).

Table 2). The total FAME from the two experiments and the two methylation methods were subsequently analyzed by ATR–IR spectroscopy, which has been applied to rapid quantitation of total *trans* content in food products (45,46). The IR spectra (Fig. 4) showed absorbances due to the *trans* monounsaturated fatty acids and methylene-interrupted and noninterrupted 18:2 at 966 cm^{-1} and absorbances due to "*trans*-containing" conjugated dienes at 985 and 948 cm^{-1} (44). The IR calibration was based only on the absorptivity of methyl elaidate, which appeared to be inappropriate for the quantitation of conjugated dienes. Therefore, the absorbances at 948 and 985 cm^{-1} were subtracted before quantitation. However, even after removing the conjugated diene contribution, the remaining IR *trans* values were sometimes higher than those determined by GLC minus the conjugated dienes (Table 2). This may be due to additional unknown or overlapping *trans* isomers in the GLC chromatogram with different absorptivities than those of methyl elaidate. Until an appropriate *trans*-free reference material is found that is close to that of milk fat composition both in isolated and conjugated *trans* fatty acids, the accurate determination of total *trans* content in milk by IR will be difficult. The results in Table 2 showed that HCl methylation leads to lower *trans* values as determined by IR, due to the production of interfering material with absorbances in the *trans* region, which made these IR determinations of total trans content unreliable. This pointed to a further disadvantage of preparing total milk FAME using only HCl/methanol.

Preparation and analysis of total rumen FAME. Rumen lipids contain a complex mixture of phospho-, sphingo-, neutral-, and sterol lipids including high levels of free fatty acids, mainly from plant origin (21). The present study was undertaken to develop a method for the preparation of FAME from total rumen lipids. $NaOCH_3$/methanol was ineffective because of the high content of free fatty acid, while diazomethane methylated the free fatty acids, but it did not react with the ester lipids. Diazomethane did not isomerize the conjugated dienes or produce methoxy artifacts (Fig. 5A). On the other hand, HCl/methanol caused isomerization of the conjugated dienes, but did not form allylic methoxy derivatives (Fig. 5B). There is no explanation for this finding, but it may suggest the absence of hydroxy fatty acids in rumen lipids or an effect of free fatty acids. A combination of $NaOCH_3$ (10

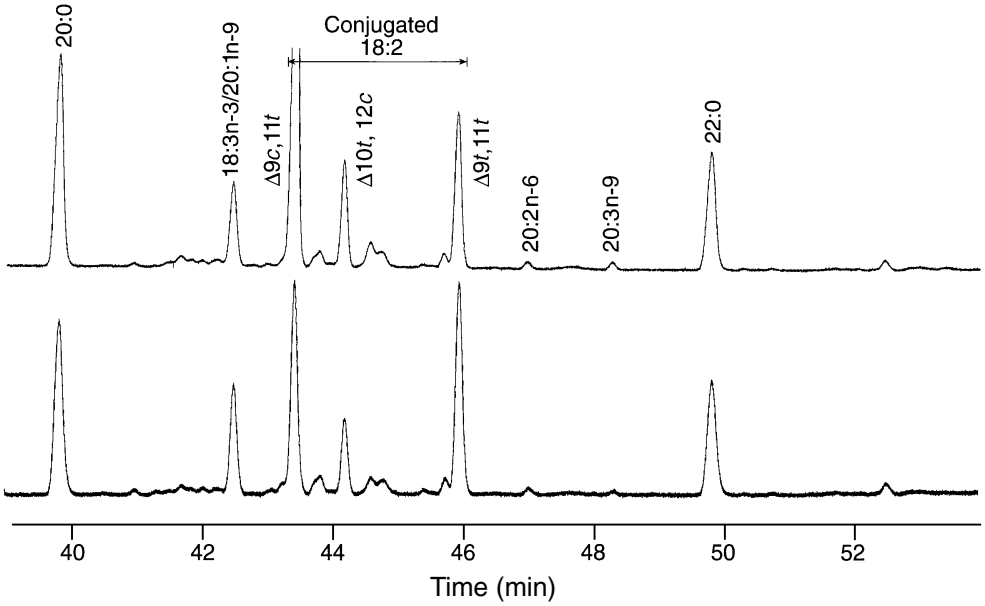

FIG. 5. The methyl eicosanoate (20:0) to methyl arachidonate (20:4n-6) region of the gas–liquid chromatogram of total rumen fatty acid methyl esters prepared by using (A) diazomethane and (B) HCl as catalysts in anhydrous methanol. Analysis was on the same type of GLC column as Figure 1. See Figure 2 for abbreviation.

min at 50°C) and HCl (10 min at 80°C) gave a Δ9*c*,11*t*-18:2 to Δ9*t*,11*t*-18:2 ratio of 4:3, while a combination of diazomethane and $NaOCH_3$ gave a ratio of 4:1. Both methods would appear to be suitable for total rumen FAME preparation. In our previous study (38), HCl/methanol was used to prepare the FAME of total rumen lipids which isomerized the conjugated dienes. However, in that study (38), only the total conjugated dienes were presented, which should be only slightly lower than if a base catalyst had been used (~12% based on milk results; Table 1).

Rumen FAME showed a similar pattern to that observed in milk FAME except for greatly reduced levels of very short-chain saturated (4:0–10:0) and long-chain C_{20} and C_{22} PUFA, and a higher content of *trans*-18:1 isomers and long-chain (22:0 to 26:0) saturated fatty acids (compare with Refs. 38 and 39). A comparison of the content of conjugated dienes suggests that it was twice as high in milk (present study, Table 1) than in rumen samples (39), both in control (0.7 vs. 0.3%) and ionophore treatments (1.4 vs. 0.7%), respectively. The content of Δ9*c*,11*t*-18:2 found in control milk (0.7%) agrees well with the value obtained by others (3) when a base-catalyzed method was used (0.85%).

Summary. The analysis of the conjugated dienes in milk and dairy products needs to be accurate, in view of their beneficial claims, and the assumption that only one of the isomers (Δ9*c*,11*t*-18:2) may be active in carcinogenesis and tumorigenesis (18). The evidence suggests that the formation of the conjugated dienes is enzymatic both by rumen bacteria (52–54) and in biological material as products of free radical *in vivo* lipid peroxidation (55–57). Careful analysis of the conjugated dienes in biological material, using only base-catalyzed methods that do not isomerize the conjugated diene system, has shown that Δ9*c*,11*t*-18:2 was the only isomer present (51). The presence of larger amounts of other isomers suggests subsequent isomerization during processing or improper (acid-catalyzed) methodology. There are many references in the literature reporting the content of conjugated dienes in milk and dairy products (3,4,6,8–15,24,27). Many of these studies show a high content of isomers other than Δ9*c*,11*t*-18:2 (10–13,15,27). Reinvestigation using proper techniques is needed.

Given the data presented here, a single procedure cannot simultaneously methylate all milk or rumen lipid components and avoid isomerization of conjugated dienes and the formation of allylic methoxy artifacts. Base-catalyzed methods caused no isomerization and produced no methoxy artifacts. However, $NaOCH_3$ did not methylate free fatty acids and *N*-acyl lipids, and diazomethane did not methylate esters. Tetramethylguanidine did not methylate sphingomyelin or phospholipids completely, which makes this method unsuitable for tissue lipids high in these lipids, as was reported recently (58). In addition, tetramethylguanidine gave undesirable products that extracted with hexane and interfered with the analyses of short-chain FAME, such as found in milk. Acid-catalyzed methods (HCl, BF_3, acetyl chloride, and H_2SO_4) methylated all lipid classes. However, these catalysts caused extensive isomerization of conjugated dienes, formed allylic methoxy artifacts, and are therefore not recommended by themselves for the analysis of milk and rumen lipids. A combination of methods appears to be most suitable, but that increases the loss of short-chain FAME during solvent removal or excessive handling. For milk, $NaOCH_3$-HCl (or BF_3) resulted in small losses of unconverted sphingomyelin, and minor isomerization of conjugated dienes and allylic methoxy formation, while diazomethane-$NaOCH_3$ ignored the sphingomyelin lipids. For rumen, either diazomethane-$NaOCH_3$, or $NaOCH_3$-HCl (or BF_3) gave good results. Analysis of isopropyl, instead of methyl esters, has the added advantage of not requiring the use of correction factors for FAME (24,30–32), but the isopropyl esters need to be prepared using Na isopropionate as catalyst instead of H_2SO_4/isopropanol (6,32). The determination of total isolated *trans* content in milk by IR methods was complicated by interferences from significant amounts of conjugated dienes.

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Effects of High Pressure and Temperature on Micelle Formation of Sodium Deoxycholate and Sodium Dodecylsulfate

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Pressure, as well as temperature, causes lipid phase transitions and changes the fluidity of membranes. For example, membrane intrinsic protein (Na, K)-ATPase is inhibited by increasing hydrostatic pressure, because increased pressure decreases the membrane fluidity to inhibit the conformational transition necessary for the (Na, K)-ATPase reaction (1). However, the effect of pressure on the lipid component in cell membranes of living organisms is not well known. For full understanding of this subject, studies of the behavior of natural and artificial lipids under high pressure are required.

In this study, sodium deoxycholate (DOC) and sodium dodecylsulfate (SDS) are used as a model of lipids, and critical micelle concentration (CMC) under high pressure is measured to investigate the pressure effects on the aggregation or the hydrophobic interaction of lipid molecules.

Absorbance under high pressure was measured with a thermocontrolled high-pressure photometer cell with sapphire windows (Teramecs Co., Kyoto, Japan) which is connected with a hand-type high-pressure pump and attached to a ultraviolet (UV)-visible spectrophotometer (UV-2500PC; Shimadzu Co., Kyoto, Japan).

A series of aqueous amphiphile solutions containing 48.4 μM Coomassie brilliant blue R-250 (Nacalai Tesque, Inc., Kyoto, Japan) were made according to the method of Courtney *et al.* (2), and 1.5 mL of the sample solution was introduced to the high-pressure cell and incubated for 3–10 min at a given temperature and pressure. Then, the absorbance at 596 nm was measured. It was checked for 15 min to obtain the stable value. The wavelength of maximum dye absorption shifts from 550 to 596 nm when amphiphiles form micelles. CMC was determined as the break point when the absorbance at 596 nm vs. the logarithm of amphiphile concentration was plotted on a graph.

CMC values of various amphiphiles determined by the Coomassie brilliant blue method at 25°C are compared with those of other methods including the conductivity method in Table 1. The values obtained by the dye method are in good agreement with those reported in the literature, except for a notably different value from Reference 3. Furthermore, it was confirmed that change of the dye concentration (12.1–48.4 μM) caused no change in CMC values obtained. Thus, the dye method employed in the present experiments is considered to be reliable.

TABLE 1
Critical Micelle Concentration (CMC) of Various Amphiphiles Determined at 25°C

Amphiphiles	CMC (mM)	
	Present result[a]	Literature value (ref. no.)
Sodium deoxycholate	3.11 ± 0.005	3.1 (4)
Sodium dodecylsulfate	4.59 ± 0.24	8.1 (3)[b], 5.9 (2), 5.4 (5)
Triton X-100	0.159 ± 0.002	0.2 (2), 0.22 (6)
Tween 20	0.105 ± 0.013	0.068 (2), 0.11 (5)

[a]Average of three or four measurements.
[b]Exceptionally high for unknown reason.

The CMC of DOC and SDS at various pressures up to 400 MPa and at temperatures of 20 to 45°C were determined (Fig. 1). In case of SDS, the pressure dependency at various temperatures was in fairly good agreement with published data (3), although the dye method tended to give somewhat lower values than the conductivity method. At any temperature, pressure dependency showed a convex curve with maximum at 100 MPa as already reported with SDS (7). The CMC of DOC increased slightly up to 100 MPa and then decreased at 100 MPa or higher at 25, 35, and 45°C (Fig. 1). However, a strong decrease in the CMC was observed at 100 MPa or

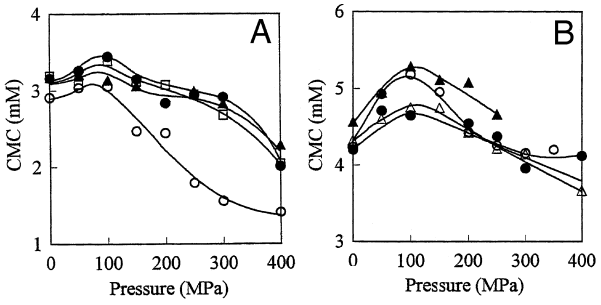

FIG. 1. Effects of pressure on critical micelle concentration (CMC) of (A) sodium deoxycholate and (B) sodium dodecylsulfate at various temperatures. ○: 20; ●: 25; △: 30; ▲: 35; □: 45°C.

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Abbreviations: CMC, critical micelle concentration; DOC, sodium deoxycholate; SDS, sodium dodecylsulfate.

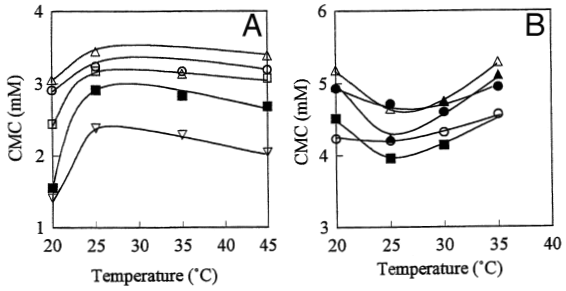

FIG. 2. Effects of temperature on critical CMC of (A) sodium deoxycholate and (B) sodium dodecylsulfate at various pressures. ○: 0.1; ●: 50; △: 100; ▲: 150; □: 200; ■: 300; ▽: 400 MPa.

higher pressures at 20°C. These results indicate that aggregation of DOC and SDS is dependent on the pressure. It may be expressed that molecular interaction of DOC and SDS decreased up to 100 MPa and increased over 100 MPa. It should be noted, however, that the CMC of SDS changed more than that of DOC when pressure changed from 0.1–100 MPa (Fig. 1).

The temperature dependence of the CMC of DOC and SDS at different pressures is shown in Figure 2. As a whole, the CMC of DOC increased up to 25°C and then decreased slightly over 25°C at any pressure, although such change was marked at higher pressure. In contrast to CMC of DOC, the temperature-dependent change of CMC of SDS showed a concave curve with a minimum at 25°C (Fig. 2). These results indicate that molecular aggregation of DOC decreased up to 25°C and increased at higher temperatures, but the aggregation of SDS increased and then decreased at 25°C.

The different behavior observed between DOC and SDS may result from the different structure of these compounds; DOC has a cyclic hydrocarbon structure with two hydroxyl groups which form hydrogen bonds in addition to the hydrophobic bonding due to the rest of molecule, whereas SDS has a linear hydrocarbon chain which makes only hydrophobic bonds. Pressure and temperature effects on hydrogen bonding and hydrophobic interaction are complicated depending on the structural properties. Therefore, complete understanding of the present results awaits systematic study of a series of lipids and of other properties including micellar radius (8,9).

ACKNOWLEDGMENTS

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Isomers in Commercial Samples of Conjugated Linoleic Acid

Sir:

In connection with our analytical activities (MRS Lipid Analysis Unit), we have analyzed several commercial samples of conjugated linoleic acid (CLA). Most of these have been prepared by alkali-isomerization of linoleic acid or of oils, such as sunflower or safflower, rich in this acid. (i) We have examined the methyl esters, which must be prepared from the acids avoiding the use of acidic catalysts, by gas chromatography (GC). With a 25-m carbowax capillary column, most of the samples show two major peaks which are well-resolved from each other, along with minor peaks running later which are first the all-*cis* and then the all-*trans* dienes. The two major peaks are usually considered to be only the 9*c*,11*t* and 10*t*,12*c* isomers. This interpretation is not consistent with the GC/mass spectrometry (MS) data reported below. When examined on a highly polar 100-m capillary column (CP Sil 88), the GC trace is more complex. Several samples show a new peak for 11,13 diene, and some indicate the presence of several other isomers. We also have evidence that the 9,11 peak contains the 8,10 isomer though we have been unable to resolve these. (ii) GC–MS of the diene adducts formed through reaction with 4-methyl-2,3,4-triazoline-3,5-dione (MTAD derivatives), with selected ion monitoring, shows the presence of 8,10; 9,11; 10,12; and 11,13 dienes (1). For example, Figure 1 illustrates the selected ion chromatogram for one of the diagnostic ions from each isomer in a commercial CLA preparation. It is clear that four unresolved isomers are present. The proportions of these vary widely (presumably depending on the conditions of alkali-isomerization) and even more isomers are sometimes present. GC–MS of the dimethyloxazoline derivatives confirmed the identity of the major products. (iii) High-resolution ^{13}C nuclear magnetic resonance spectroscopy confirmed the presence of at least four *cis,trans* conjugated dienes and gave quantitative results in line with those obtained by GC–MS analysis.

We conclude that most samples of CLA, though rich in the 9,11 (probably mainly if not entirely the 9*c*,11*t* isomer) and 10,12 dienes (probably mainly if not entirely the 10*t*,12*c* isomer) also contain at least the 8,10 and 11,13 *cis,trans* dienes, sometimes at quite high levels. These are accompanied by all-*cis* and all-*trans* dienes. In one commercial sample of CLA, we found the following dienes: 8,10 (14%), 9,11 (30%), 10,12 (31%), and 11,13 (24%).

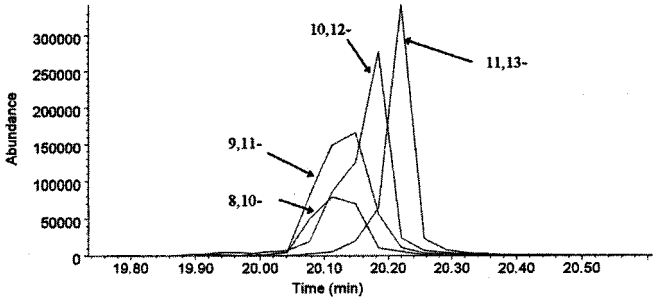

FIG. 1. Selected ion scan in gas chromatography–mass spectrometry of methyl triazoline dione adducts of a commercial conjugated linoleic acid preparation (methyl esters). The characteristic ions are at *m/z* = 264 (8,10–18:2), 250 (9,11–18:2), 236 (10,12–18:2), and 222 (11,13–18:2).

It is important that those who produce these materials and use them for research purposes appreciate the complex nature of their products. To our knowledge, the identity of the biologically active CLA is not known although it is generally assumed to be 9*c*,11*t*-18:2. Nor is it known how the activity of this isomer may be influenced by the presence of other isomeric CLA.

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Fatty Acyl Desaturation in Isolated Hepatocytes from Atlantic Salmon (*Salmo salar*): Stimulation by Dietary Borage Oil Containing γ-Linolenic Acid

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ABSTRACT: The effects of different dietary oils on the fatty acid compositions of liver phospholipids and the desaturation and elongation of [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6 were investigated in isolated hepatocytes from Atlantic salmon. Atlantic salmon smolts were fed diets containing either a standard fish oil (FO) as a control diet, a 1:1 blend of Southern Hemisphere marine oil and tuna orbital oil (MO/TO), sunflower oil (SO), borage oil (BO), or olive oil (OO) for 12 wk. The SO and BO diets significantly increased the percentages of 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, and total n-6 polyunsaturated fatty acids (PUFA) in salmon liver lipids in comparison with the FO diet. The BO diet also increased the percentage of 20:4n-6. Both the SO and BO diets significantly reduced the percentages of all n-3 PUFA in comparison with the FO diet. The OO diet significantly increased the percentages of 18:1n-9, 18:2n-6, total monoenes, and total n-6 PUFA in liver lipids compared to the FO diet, and the percentages of all n-3 PUFA were significantly reduced. With [1-^{14}C]18:3n-3, the recovery of radioactivity in the products of Δ6 desaturation was significantly greater in the hepatocytes from salmon fed SO, BO, and OO in comparison with the FO diet. The BO diet also increased the recovery of radioactivity in the products of Δ5 desaturation. Only the BO diet significantly affected the desaturation of [1-^{14}C]18:2n-6, increasing recovery of radioactivity in both Δ6- and Δ5-desaturation products. In conclusion, dietary BO, enriched in γ-linolenic acid (18:3n-6), significantly increased the proportions of both 20:3n-6 and 20:4n-6 in salmon liver phospholipids and also significantly increased the desaturation of both 18:2n-6 and 18:3n-3 in salmon hepatocytes. The possible relationships between dietary fatty acid composition, tissue phospholipid fatty acid composition, and desaturation/elongation activities are discussed.

Lipids 32, 1237–1247 (1997).

The fatty acid compositions of animal tissues are determined both by the type of dietary lipid ingested and the ability of the individual species to modify that dietary input *via* both catabolism and by pathways of desaturation and elongation (1–4). Both the fatty acid-desaturating and -elongating enzyme systems are microsomal, consisting of electron transport chains linked to terminal desaturase and elongase activities (5–7). As such, they represent membrane-bound systems whose activities, like all membrane-bound enzymes and receptors, are highly dependent upon membrane fatty acid compositions (8–11). Therefore, hepatic desaturation and elongation of fatty acids are modulated by nutritional state including the content and composition of dietary lipids and fatty acids (5,6). For instance, fish oil-containing diets, rich in eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA), tended to reduce rat liver microsomal Δ6-desaturase activity, particularly with 18:2n-6 as substrate, in comparison with diets rich in 18:2n-6 or 18:3n-3 (12–14). However, fatty acyl desaturase activities will themselves alter cellular fatty acid compositions, and it is unclear what precise relationship exists between dietary fatty acids, fatty acid desaturation, and tissue fatty acid compositions.

Freshwater fish are able to elongate and desaturate 18:3n-3 to 22:6n-3, whereas marine fish lack or have a very low activity of Δ5 desaturase and so require the long-chain polyunsaturated fatty acids (PUFA), EPA and DHA, to be supplied by the diet (15–17). Dietary specialization is an important factor in determining this difference since many freshwater fish are herbivorous or omnivorous, whereas the limited number of marine fish studied to date are predominantly carnivorous (17,18). Therefore, in terms of desaturase activities, freshwater fish are similar to terrestrial omnivores such as rodents and humans, but marine fish are more similar to terrestrial carnivores such as cats, which have relatively low desaturase activities (19–21). Atlantic salmon (*Salmo salar*) is an anadromous fish which begins life in freshwater and migrates to the sea after 1–2 yr, before returning to freshwater to breed after spending 1–3 yr at sea. Like other salmonids, such as trout, the desaturase profile of salmon appears to be that of freshwater fish (22,23). However, the commercial culture of most fish species, including salmon, is currently highly dependent on diets containing fish oil and fish meal which are increasingly expensive and whose supply is both volatile/variable

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Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; BHT, butylated hydroxytoluene; BO, borage oil diet; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAF-BSA, fatty acid-free bovine serum albumin; FAME, fatty acid methyl esters; FO, standard Northern Hemisphere fish oil diet; GLA, γ-linolenic acid; HBSS, Hanks' balanced salt solution; MO, marine oil; OO, olive oil diet; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid(s); SO, sunflower oil diet; TLC, thin-layer chromatography; TO, tuna orbital oil diet.

and becoming increasingly environmentally undesirable owing to the overexploitation of unsustainable marine fish stocks (24). Fish oils are also in increasing demand as direct sources of n-3 PUFA for human nutrition (25). Several studies have investigated the use of other oils in the nutrition of salmon (25–27). Different dietary oils have been shown to significantly alter tissue phospholipid fatty acid compositions in salmon (28,29) and have significant effects on functional activities including phospholipase A activity (30), eicosanoid metabolism (31), and immune function (32). However, despite the importance of understanding the relationship between dietary fatty acids, tissue fatty acid compositions and fatty acyl desaturation, there are few data on the effects of dietary oils and fatty acids in modulating desaturase activities in fish and none on the commercially important salmon (33,34).

In the present study, we investigated the effects of different dietary oils on the desaturation of both [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6 in isolated hepatocytes from Atlantic salmon. Because the long-chain n-3 PUFA, EPA, and DHA are known to be inhibitory to fatty acyl desaturases (5,19), oils were chosen to alter tissue EPA and DHA levels. With a standard fish oil diet as the control diet, one diet contained a blend of Southern Hemisphere fish oil (high EPA) and tuna orbital oil (high DHA) to increase EPA and DHA. Other diets were formulated to reduce cellular EPA and DHA by using combinations of 18:1n-9, 18:2n-6, and 18:3n-6. The results showed that dietary borage oil (BO), enriched in γ-linolenic acid (GLA; 18:3n-6), significantly increased the proportions of both 20:3n-6 and 20:4n-6 in salmon liver phospholipids and also significantly increased the desaturation of both 18:2n-6 and 18:3n-3 in salmon hepatocytes.

MATERIALS AND METHODS

Animals and diets. Five hundred fifty Atlantic salmon S1 smolts (initial mean weight 38 g) were obtained from the S.O.A.E.F.D. Fish Cultivation Unit (Aultbea, Wester Ross, Scotland) and distributed randomly into five tanks of 500-L capacity each, which were supplied with seawater at a rate of 10 L/min. The fish were subject to natural photoperiod, and the water temperature during the experimental period (July–October) varied from 9–17°C. Diets were fed for 12 wk and were supplied by automatic feeders which were adjusted to supply 25 g/kg biomass/day. Fish were weighed individually at the start and finish of the experiment and weighed in bulk every 28 d and the ration adjusted accordingly. The experiment was conducted in accordance with the British Home Office guidelines regarding research on experimental animals.

The diets were formulated to satisfy the nutritional requirements of salmonid fish and contained 48% crude protein and 25% lipid (35). The exact formulation of the diets is shown in Table 1. All diets were prepared to order by Ewos Ltd. (Technology Centre, Livingston, United Kingdom). Pellets, containing 3% fish oil, were manufactured by extrusion, and experimental oils (160 g/kg) were then added after addition of an antioxidant mix (0.4 g/kg; composition as described in Table 1) to each oil before mixing with the other ingredients. The dietary oils were a standard Northern Hemisphere fish oil (FO) (Fosol; Seven Seas Ltd., Hull, United Kingdom) as a control diet, a 1:1 mix of a Southern Hemisphere fish oil [marine oil (MO)] (Marinol K; Fishing Industry Research Unit, Rosebank, Republic of South Africa) and DHA-rich tuna orbitol oil (TO) (Croda Universal Ltd., Hull, United Kingdom), sunflower oil (SO) (Tesco Ltd., Cheshunt, United Kingdom), borage oil (BO) (Croda Universal Ltd.), and olive oil (OO) (Tesco Ltd.). The fatty acid compositions of the five diets are shown in Table 2.

TABLE 1
Formulation of Diets[a]

Component	Content (g/kg)
Fish meal[b]	620
Soya meal (full fat)	80
Wheat meal	66
Mineral mix[c]	24
Vitamin mix[d]	10
Choline chloride (40% wt/vol)	10 mL (4 g)
Feeding stimulant[e]	6
Fish Oil[f]	30
Oil coating[g]	160

[a]Diets were manufactured to order by Ewos Ltd., Technology Centre, Livingston, United Kingdom. Base pellets containing 3% fish oil were prepared by extrusion. Experimental oils were then added to the base pellets.
[b]LT94, Low-temperature fish meal (Ewos Ltd., Bathgate, United Kingdom).
[c]Supplied (per kg diet): KH_2PO_4, 22 g; $FeSO_4 \cdot 7H_2O$, 1.0 g; $ZnSO_4 \cdot 7H_2O$, 0.13 g; $MnSO_4 \cdot 4H_2O$, 52.8 mg; $CuSO_4 \cdot 5H_2O$,12 mg; $CoSO_4 \cdot 7H_2O$, 2 mg; and KI, 2 mg.
[d]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.
[e]Finnstim (Ewos Ltd., Bathgate, United Kingdom).
[f]Norsalmoil (United Fish Products, Aberdeen, United Kingdom).
[g]The experimental oils were: A control diet containing a standard Northern Hemisphere fish oil (Fosol; Seven Seas Ltd., Hull, United Kingdom); a 1:1 mix of a Southern Hemisphere fish oil (Marinol K; Fishing Industry Research Unit, Rosebank, Republic of South Africa) and docosahexaenoic acid-rich tuna orbital oil (Croda Universal Ltd., Hull, United Kingdom); sunflower oil (Tesco Ltd., Cheshunt, United Kingdom); borage oil (Croda Universal Ltd.); olive oil (Tesco Ltd.). All experimental oils had antioxidant mix [dissolved in propylene glycol and containing (g/L): butylated hydroxyanisole, 60; propyl gallate, 60; citric acid, 40] added prior to coating.

Lipid extraction, glycerophospholipid separation, and fatty acid analysis. Fish were killed by a blow to the head and livers were dissected from four fish per dietary treatment and immediately frozen in liquid nitrogen. Samples were stored at −40°C prior to lipid extraction. Total lipid was extracted from liver and diet samples by the method of Folch *et al.* (36). Samples of liver total lipid (3–4 mg) were applied to a 4-cm origin on a thin-layer chromatography (TLC) plate, and the polar lipid classes were separated using methyl acetate/propan-2-ol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol) as developing solvent (37). Lipid

TABLE 2
Fatty Acid Compositions (wt%) of Diets[a]

	Diet				
Fatty acid	Fosol	Tuna oil/Marinol	Sunflower oil	Borage oil	Olive oil
14:0	4.9	4.8	2.0	1.6	1.5
16:0	14.4	16.0	9.8	11.9	12.9
18:0	2.5	3.3	3.6	3.4	3.1
Total saturates	22.9	25.4	16.5	17.5	18.4
16:1n-7	4.9	6.3	1.8	1.5	2.0
18:1n-9	14.4	12.5	18.2	16.3	46.8
18:1n-7	2.5	2.5	1.3	1.2	2.0
20:1n-9	8.7	4.6	3.1	5.2	3.4
22:1n-11	12.4	6.1	3.8	3.7	3.7
22:1n-9	0.3	0.3	0.2	1.6	0.4
24:1	0.9	0.6	0.3	1.1	0.4
Total monoenes	44.2	33.9	28.7	30.6	58.7
18:2n-6	6.0	5.6	46.4	31.7	14.2
18:3n-6	0.1	0.2	trace	12.5	0.2
20:2n-6	0.2	0.2	trace	0.2	0.1
20:3n-6	trace	trace	n.d.	n.d.	n.d.
20:4n-6	0.5	1.1	0.2	0.2	0.1
22:5n-6	0.2	0.5	trace	trace	trace
Total n-6 PUFA	7.0	7.6	46.6	44.6	14.6
18:3n-3	1.6	1.2	0.9	0.9	1.1
18:4n-3	2.6	2.0	0.8	0.8	0.6
20:4n-3	0.7	0.6	0.2	0.2	0.1
20:5n-3	6.0	9.5	2.6	2.1	1.7
22:5n-3	1.1	1.2	0.3	0.2	0.2
22:6n-3	8.8	12.6	3.7	3.2	2.7
Total n-3 PUFA	20.8	27.1	8.3	7.4	6.4
C16 PUFA	0.9	2.0	trace	trace	trace
Total PUFA	27.8	34.7	54.9	52.0	21.0
n-3/n-6	3.0	3.6	0.2	0.2	0.4

[a]n.d., not detected; trace value, < 0.05%; PUFA, polyunsaturated fatty acids; Fosol (Seven Seas Ltd., Hull, United Kingdom); Marinol (Fishing Industry Research Unit, Rosebank, Republic of South Africa).

classes were visualized under ultraviolet light after spraying with 2,7-dichlorofluorescein. Individual glycerophospholipid classes were scraped into stoppered test tubes and transmethylated directly on the silica after addition of 1 mL toluene and 2.5 mL 1% sulfuric acid in methanol, and fatty acid methyl esters (FAME) prepared as described below. FAME were prepared from total lipid and individual glycerophospholipid classes by acid-catalyzed transmethylation at 50°C for 16 h (38) and were extracted and purified as described previously (39). FAME were analyzed in a Packard 436 gas chromatograph (Canberra Packard, Pangbourne, United Kingdom) equipped with a CP Wax 52CB fused-silica capillary column (30 m × 0.32 mm i.d., Chrompack U.K. Ltd., London, England), with on-column injection using hydrogen as carrier gas and a biphasic thermal gradient from 50 to 225°C. FAME were identified by comparison with known standards and a well-characterized fish oil and by reference to published data as described previously (39) and were quantified using a Shimadzu CR6A data processor (Kyoto, Japan). All solvents contained 0.01% butylated hydroxytoluene (BHT) as an antioxidant.

Preparation of isolated hepatocytes. Intact, metabolically viable liver parenchymal cells were prepared by the technique of *in vitro* collagenase dispersion essentially as described in detail previously (23). Briefly, fish were killed by a blow to the head and the liver dissected immediately. The gall bladder was removed carefully from the liver and the main blood vessels removed. The liver was perfused *via* the hepatic vein with solution A [calcium- and magnesium-free Hanks' balanced salt solution (HBSS) + 10 mM HEPES + 1 mM EDTA], using a syringe fitted with a 23-gauge needle, to clear blood from the tissue. The liver was chopped finely with scissors and incubated with 20 mL of solution A containing 0.1% (wt/vol) collagenase in a 25 mL "Reacti-flask" in a shaking water bath at 20°C for 45 min. The digested liver was filtered through 100-μm nylon gauze and the cells collected by centrifugation at 1000 × *g* for 5 min. The cell pellet was washed with 20 mL of solution A containing 1% wt/vol fatty acid-free bovine serum albumin (FAF-BSA) and recentrifuged. The hepatocytes were resuspended in 10 mL of Medium 199 (Sigma Chemical Co., Poole, United Kingdom) containing 10 mM HEPES, 2 mM glutamine, 100 U/mL penicillin, and 0.1

mg/mL streptomycin. One hundred μL of cell suspension was mixed with 400 μL of Trypan Blue, and hepatocytes were counted and their viability assessed using a hemacytometer. One hundred μL of the cell suspension was retained for protein determination.

Preparation of isotopes for addition to cell cultures. The radioactive isotopes, [1-^{14}C]18:3n-3 or [1-^{14}C]18:2n-6, were added to cells as complexes with FAF-BSA. Briefly, 25 μCi of fatty acid (0.5 μmol) in ethanol was placed in a reaction vial, solvent was evaporated under a stream of nitrogen, and 100 μL of 0.1M KOH were added. The mixture was stirred for 10 min at room temperature before 5 mL of 50 mg/mL FAF-BSA in HBSS containing 10 mM HEPES buffer was added and the mixture stirred for 45 min at 20°C.

Assay of hepatocyte fatty acyl desaturation/elongation activities. Five mL of each hepatocyte suspension was dispensed into two 25-cm^2 tissue culture flasks. Hepatocytes were incubated with 0.25 μCi of either [1-^{14}C]18:3n-3 or [1-^{14}C]18:2n-6, added as complexes with FAF-BSA prepared as described above. After addition of isotope, the flasks were incubated at 20°C for 3 h. After incubation, the cell layer was dislodged by gentle rocking, transferred to glass conical test tubes, and the flasks were washed with 1 mL of ice-cold HBSS containing 1% FAF-BSA. The cell suspensions were centrifuged at 300 × *g* for 2 min, the supernatants discarded, and the cell pellets washed with 5 mL of ice-cold HBSS/FAF-BSA. The supernatants were discarded and the tubes placed upside down on paper towels to blot for 15 s before extraction of total lipid using ice-cold chloroform/methanol (2:1, vol/vol) containing 0.01% (wt/vol) BHT essentially as described by Folch *et al.* (36) and as detailed previously (40).

Total lipid was transmethylated and FAME were prepared as described above. The methyl esters were redissolved in 100 μL hexane containing 0.01% BHT and applied as 2.5-cm streaks to TLC plates impregnated by spraying with 2 g silver nitrate in 20 mL acetonitrile and preactivated at 110°C for 30 min. Plates were fully developed in toluene/acetonitrile (95:5, vol/vol) (41). Autoradiography was performed with Kodak Biomax MR-2 film for 4–7 d at room temperature. Silica corresponding to individual PUFA was scraped into scintillation minivials containing 2.5 mL of scintillation fluid (Ecoscint A. National Diagnostics, Atlanta, GA) and radioactivity determined in a TRI-CARB 2000CA scintillation counter (United Technologies Packard, Pangbourne, United Kingdom). Results were corrected for counting efficiency and quenching of ^{14}C under exactly these conditions.

Protein determination. Protein concentration in isolated hepatocyte suspensions was determined according to the method of Lowry *et al.* (42) after incubation with 0.25 mL of 0.25% (wt/vol) SDS/1 M NaOH for 45 min at 60°C.

Materials. [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6 (both 50–55 mCi/mmol) were from NEN [DuPont (U.K.) Ltd., Stevenage, United Kingdom)]. HBSS, HEPES buffer, glutamine, penicillin, streptomycin, collagenase (type IV), FAF-BSA, BHT, and silver nitrate were obtained from Sigma Chemical Co. TLC plates, precoated with silica gel 60 (without fluorescent indicator), were obtained from Merck (Darmstadt, Germany). All solvents were HPLC-grade and were obtained from BS & S Ltd. (Edinburgh, Scotland).

Statistical analysis. Significance of difference between dietary treatments ($P < 0.05$) was determined by analysis of variance (ANOVA). Analyses were performed using a Statgraphics (system 3.0) computer package (Statistical Graphics Corp., Rockville, MD). Data which were identified as nonhomogeneous (using Bartlett's test) were subjected to either arcsin square root or log transformation before analysis. Where appropriate, differences between means were determined by Tukey's test.

RESULTS

The mean initial weight of all the fish was 37.9 ± 10.8 g, and after 12 wk on the diets the final weights of the fish were 150.0 ± 49.5 g (FO), 159.5 ± 40.0 g (TO/MO), 128.8 ± 48.2 g (SO), 137.9 ± 32.8 g (BO), and 150.5 ± 47.7 g (OO). The only difference in mean final weights that was significant (one-way ANOVA) was between the TO/MO and SO diets.

Replacing the control fish oil (Fosol; FO) with the combination of Southern Hemisphere MO and TO increased the EPA, DHA, total n-3 PUFA, and total PUFA contents of the diet at the expense of saturates (Table 2). The SO and BO both increased the total n-6 PUFA contents of the diets, owing solely to increased 18:2n-6 in SO and 18:2n-6 and 18:3n-6 in BO, at the expense of all other fatty acids compared to the control diet. The OO diet contained increased 18:1n-9, 18:2n-6, total monoenes, and total n-6 PUFA compared to the FO diet and proportions of saturated fatty acids and n-3 PUFA similar to those of the SO and BO diets (Table 2).

The MO/TO diet did not significantly affect the percentages of EPA, DHA, or total n-3 PUFA in salmon liver total lipid in comparison with the control diet (Table 3). However, the MO/TO diet significantly reduced the percentages of 20:1 and 22:1 and the n-3/n-6 ratio and increased the proportion of saturated fatty acids. The SO diet significantly increased the percentages of 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, and total n-6 PUFA in total lipid from salmon liver in comparison with the FO diet (Table 3). The BO diet had similar effects to those of SO except that it also increased the percentage of arachidonic acid (20:4n-6; AA). However, the levels of 18:2n-6 and 20:2n-6 were significantly lower in the BO-fed fish compared to the SO-fed fish, whereas the percentages of 18:3n-6, 20:3n-6, and 20:4n-6 were all significantly higher in the BO diet (Table 3). There was no difference between the SO and BO diets in the percentages of n-3 PUFA in the liver total lipid although both the SO and BO diets significantly reduced the percentages of all n-3 PUFA in comparison with the FO diet. The OO diet significantly increased the percentages of 18:1n-9, 18:2n-6, total monoenes, and total n-6 PUFA in liver total lipid compared to the FO diet, and the percentages of all n-3 PUFA were significantly reduced and similar to the levels observed in the SO- and BO-fed fish (Table 3).

The differences in specific fatty acids observed in liver total

TABLE 3
Effects of Diet on the Fatty Acid Compositions of Total Lipid from the Liver of Atlantic Salmon[a]

	Diet				
Fatty acid	Fosol	Tuna oil/Marinol	Sunflower oil	Borage oil	Olive oil
Total saturates	21.7 ± 1.0[b]	24.2 ± 0.4[a]	20.1 ± 1.7[b]	22.3 ± 1.6[a,b]	20.1 ± 0.5[b]
16:1[1]	2.5 ± 0.2[a,b]	3.0 ± 0.2[a]	1.3 ± 0.2[c]	1.7 ± 0.6[b,c]	1.7 ± 0.1[b,c]
18:1n-9	11.2 ± 0.7[b]	9.6 ± 0.3[b]	12.4 ± 1.5[b]	13.1 ± 4.1[b]	30.5 ± 1.6[a]
18:1n-7	2.2 ± 0.2[a,b]	2.3 ± 0.1[a]	1.1 ± 0.1[c]	1.1 ± 0.2[c]	1.9 ± 0.1[b]
20:1[2]	5.0 ± 0.7[a]	2.8 ± 0.3[b,c]	2.2 ± 0.4[c]	2.6 ± 0.5[c]	3.8 ± 0.2[b]
22:1n-11	2.3 ± 0.6[a]	1.3 ± 0.3[b]	1.2 ± 0.4[b]	1.1 ± 0.3[b]	1.4 ± 0.3[b]
Total monoenes	23.2 ± 2.5[b]	19.0 ± 0.7[b]	18.3 ± 2.1[b]	19.6 ± 4.9[b]	39.3 ± 1.2[a]
18:2n-6	1.9 ± 0.1[d]	2.2 ± 0.2[c,d]	22.7 ± 1.3[a]	10.9 ± 3.2[b]	5.3 ± 0.2[c]
18:3n-6	n.d.[c]	0.2 ± 0.1[c]	0.5 ± 0.1[b]	3.2 ± 0.3[a]	0.1 ± 0.1[c]
20:2n-6	0.6 ± 0.1[b]	0.4 ± 0.1[b]	3.3 ± 1.3[a]	1.0 ± 0.3[b]	0.9 ± 0.1[b]
20:3n-6	0.2 ± 0.0[d]	0.3 ± 0.1[d]	3.3 ± 0.7[b]	7.0 ± 0.6[a]	1.6 ± 0.1[c]
20:4n-6	2.9 ± 0.2[b]	3.3 ± 0.2[b]	3.5 ± 0.4[a,b]	5.9 ± 2.4[a]	1.8 ± 0.1[b]
22:4n-6	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.5 ± 0.5	n.d.
22:5n-6	0.5 ± 0.0[a,b]	0.8 ± 0.1[a,b]	0.4 ± 0.1[b]	1.3 ± 0.3[a]	0.3 ± 0.1[b]
Total n-6 PUFA	6.2 ± 0.2[d]	7.3 ± 0.2[c,d]	33.9 ± 2.1[a]	29.8 ± 2.1[b]	10.0 ± 0.2[c]
18:3n-3	0.6 ± 0.1[a]	0.5 ± 0.1[a]	0.2 ± 0.1[b,c]	0.2 ± 0.1[c]	0.3 ± 0.1[b]
18:4n-3	0.4 ± 0.1[a]	0.4 ± 0.1[a]	0.1 ± 0.1[b]	0.2 ± 0.0[a,b]	0.2 ± 0.0[a,b]
20:3n-3	0.2 ± 0.1[a]	0.1 ± 0.1[b]	n.d.[c]	n.d.[c]	n.d.[c]
20:4n-3	1.1 ± 0.2[a]	0.7 ± 0.1[b]	0.3 ± 0.1[c,d]	0.2 ± 0.1[d]	0.4 ± 0.1[c]
20:5n-3	6.7 ± 0.3[a]	7.0 ± 0.9[a]	2.6 ± 0.3[c]	2.8 ± 0.8[c]	4.0 ± 0.3[b]
22:5n-3	2.6 ± 0.2[a]	2.5 ± 0.2[a]	1.2 ± 0.2[b]	1.3 ± 0.3[b]	1.3 ± 0.2[b]
22:6n-3	34.4 ± 1.1[a]	35.4 ± 1.2[a]	21.9 ± 2.0[b]	23.6 ± 4.3[b]	23.8 ± 0.8[b]
Total n-3 PUFA	46.0 ± 1.1[a]	46.6 ± 0.6[a]	26.3 ± 2.5[b]	28.3 ± 5.2[b]	30.0 ± 1.0[b]
Total PUFA[3]	53.2 ± 1.3[c]	54.8 ± 0.7[b,c]	60.7 ± 1.6[a]	58.6 ± 3.9[a,b]	40.5 ± 0.9[d]
n-3/n-6	7.4 ± 0.2[a]	6.4 ± 0.2[b]	0.8 ± 0.2[d]	1.0 ± 0.3[d]	3.0 ± 0.2[c]

[a]Results are expressed as percentage of weight and are means ± SD ($n = 4$). The significance of differences between the dietary treatments was analyzed by one-way analysis of variance. Mean values with different superscript letters within a given row are significantly different ($P < 0.05$) as determined by Tukey's multiple range test. [1], Predominantly n-7 isomer; [2], predominantly n-9 isomer; [3], includes C_{16} and n-9 PUFA present in all samples at percentages varying from 0.3 to 0.6 and 0.2 to 0.4, respectively; n.d., not detected; PUFA, polyunsaturated fatty acids. See Table 2 for company sources.

lipid were associated with specific phospholipid classes related to the unique fatty acid compositions of the individual phospholipid classes (Tables 4–7). Therefore, in the fish fed the SO diet which, in particular, significantly increased the percentages of 18:2n-6 and 20:2n-6, the increases were most prominent in phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (Tables 4–6). With the BO diet, which showed the highest 18:3n-6 and 20:3n-6 in liver total lipid, the increased percentage of 18:3n-6 was mainly found in PC, whereas the increased percentages of 20:3n-6 were found in both PC and PE (Tables 4,5). The level of 20:4n-6 was significantly increased by both the SO and BO diets in PC and PE (Tables 4,5). However, the percentages of 20:4n-6 were greatest in phosphatidylinositol which was also the class most resistant to dietary effects with only the OO diet resulting in a significantly lower level of 20:4n-6 in phosphatidylinositol (Table 7).

Two-way ANOVA showed that less radioactivity was recovered unmetabolized with [1-^{14}C]18:3n-3 as substrate compared to [1-^{14}C]18:2n-6, whereas significantly more 18:2n-6 was metabolized by elongation to the so-called "dead-end product," 20:2n-6, compared to the similar product, 20:3n-3, arising by elongation of 18:3n-3 (Table 8). The amount of radioactivity recovered as either 18:3n-3 or 18:2n-6 and, therefore, unmetabolized, as a percentage of the total radioactivity recovered in PUFA was significantly lower in fish fed BO compared to fish on any of the other diets (Table 8). There was no significant difference between the fish on the FO, TO/MO, SO, and OO diets in the amount of radioactivity recovered unmetabolized as either 18:3n-3 or 18:2n-6. The percentage of radioactivity recovered in the elongation products, 20:3n-3 and 20:2n-6, was significantly lower in BO-fed fish compared to fish fed the diets containing fish oils, FO and TO/MO (Table 8). There was no significant difference between the fish on the FO, TO/MO, SO, and OO diets in the amount of radioactivity recovered as either 20:3n-3, but the OO diet significantly reduced the recovery of radioactivity in 20:2n-6 compared to the diets containing fish oil.

The total amount of desaturation of [1-^{14}C]18:3n-3 was significantly greater in the hepatocytes from salmon fed the n-6 PUFA-rich oils and OO in comparison with both the diets containing fish oil (Table 9). Both the n-6 PUFA-rich oils and OO increased the amount of radioactivity recovered in the Δ6 products compared to the fish oil diets, whereas the BO diet also markedly increased the recovery of radioactivity in the

TABLE 4
Effects of Diet on the Fatty Acid Compositions of Phosphatidylcholine from the Liver of Atlantic Salmon[a]

	Diet				
Fatty acid	Fosol	Tuna oil/Marinol	Sunflower oil	Borage oil	Olive oil
Total saturates	31.3 ± 1.4	33.7 ± 1.9	30.6 ± 2.7	32.5 ± 1.1	31.7 ± 0.7
Total monoenes	15.3 ± 0.8^{b}	12.6 ± 0.9^{c}	12.2 ± 0.9^{c}	12.8 ± 0.8^{c}	24.8 ± 1.2^{a}
18:2n-6	1.1 ± 0.1^{d}	0.9 ± 0.2^{d}	15.2 ± 0.9^{a}	8.0 ± 0.7^{b}	4.5 ± 0.4^{c}
18:3n-6	n.d.^{b}	n.d.^{b}	0.2 ± 0.0^{b}	2.6 ± 0.5^{a}	0.1 ± 0.0^{b}
20:2n-6	0.5 ± 0.1^{b,c}	0.3 ± 0.1^{c}	3.4 ± 0.6^{a}	0.8 ± 0.3^{c}	1.1 ± 0.2^{b}
20:3n-6	0.1 ± 0.1^{d}	0.2 ± 0.1^{d}	3.8 ± 0.5^{b}	6.8 ± 0.9^{a}	1.6 ± 0.2^{c}
20:4n-6	1.0 ± 0.0^{b,c}	1.3 ± 0.2^{b,c}	1.8 ± 0.3^{a,b}	2.6 ± 0.9^{a}	0.6 ± 0.1^{c}
22:4n-6	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.2	0.1 ± 0.0
22:5n-6	0.5 ± 0.1^{a,b}	0.8 ± 0.2^{a}	0.4 ± 0.1^{a,b}	0.6 ± 0.2^{a,b}	0.4 ± 0.1^{b}
Total n-6 PUFA	3.5 ± 0.2^{d}	3.6 ± 0.2^{d}	25.0 ± 1.5^{a}	21.6 ± 1.5^{b}	8.4 ± 0.5^{c}
18:3n-3	0.2 ± 0.1^{a}	0.2 ± 0.1^{a,b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.2 ± 0.0^{a}
18:4n-3	0.2 ± 0.0^{a}	0.1 ± 0.1^{a,b}	trace^{b}	0.2 ± 0.1^{a,b}	0.1 ± 0.1^{a,b}
20:3n-3	0.2 ± 0.1^{a}	0.1 ± 0.1^{b}	n.d.^{c}	n.d.^{c}	n.d.^{c}
20:4n-3	0.7 ± 0.1^{a}	0.4 ± 0.1^{b}	0.2 ± 0.0^{c}	0.2 ± 0.0^{c}	0.4 ± 0.1^{b}
20:5n-3	7.0 ± 0.3^{a}	6.7 ± 1.4^{a}	2.8 ± 0.5^{b}	3.3 ± 0.5^{b}	4.3 ± 0.6^{b}
22:5n-3	2.2 ± 0.2^{a}	1.9 ± 0.2^{a,b}	1.2 ± 0.2^{c}	1.3 ± 0.2^{c}	1.4 ± 0.3^{b,c}
22:6n-3	35.6 ± 0.6^{a}	38.0 ± 2.7^{a}	25.6 ± 0.4^{b}	26.2 ± 0.8^{b}	27.1 ± 2.1^{b}
Total n-3 PUFA	45.9 ± 0.4^{a}	47.3 ± 2.0^{a}	29.9 ± 0.6^{b}	31.3 ± 0.9^{b}	33.5 ± 2.0^{b}
Total PUFA	50.2 ± 0.5^{b}	51.6 ± 1.9^{a,b}	55.3 ± 2.1^{a}	53.3 ± 1.6^{a,b}	42.2 ± 1.6^{c}
n-3/n-6	13.7 ± 0.8^{a}	13.5 ± 1.1^{a}	1.2 ± 0.1^{c}	1.5 ± 0.1^{c}	4.0 ± 1.6^{b}

[a]See Table 3 footnote; trace value, < 0.05%. See Table 2 for company sources.

Δ5 products, which was 2.6-fold greater than with any other diet, and the Δ6* products (hexaenes, predominantly 22:6n-3; formerly termed Δ4 products). Two-way ANOVA showed that as well as the significant effects of diet, there was a very significant effect of substrate with significantly greater desaturation activity toward [1-^{14}C]18:3n-3 compared to [1-^{14}C]-18:2n-6 with significantly greater recovery of radioactivity in the Δ6-, Δ5- and total-desaturation products (Table 9). The

TABLE 5
Effects of Diet on the Fatty Acid Compositions of Phosphatidylethanolamine from the Liver of Atlantic Salmon[a]

	Diet				
Fatty acid	Fosol	Tuna oil/Marinol	Sunflower oil	Borage oil	Olive oil
Total saturates	16.2 ± 0.8^{a,b}	18.2 ± 0.5^{a}	16.3 ± 1.9^{a,b}	16.8 ± 0.3^{a,b}	15.2 ± 0.6^{b}
Total monoenes	22.2 ± 0.8^{b}	17.8 ± 0.7^{c}	11.6 ± 0.7^{e}	14.1 ± 0.3^{d}	25.9 ± 0.6^{a}
18:2n-6	1.4 ± 0.2^{c}	2.1 ± 0.3^{c}	13.3 ± 1.4^{a}	9.7 ± 0.3^{b}	3.2 ± 0.2^{c}
18:3n-6	0.2 ± 0.1^{b,c}	0.2 ± 0.0^{b}	n.d.^{c}	0.4 ± 0.2^{a}	n.d.^{c}
20:2n-6	0.9 ± 0.0^{b}	0.7 ± 0.1^{b}	4.3 ± 0.7^{a}	1.2 ± 0.5^{b}	1.0 ± 0.1^{b}
20:3n-6	0.1 ± 0.1^{d}	0.2 ± 0.1^{d}	4.0 ± 0.5^{b}	6.3 ± 0.7^{a}	1.6 ± 0.1^{c}
20:4n-6	1.6 ± 0.1^{b}	2.3 ± 0.2^{b}	5.0 ± 0.7^{a}	5.8 ± 1.4^{a}	1.5 ± 0.1^{b}
22:4n-6	0.1 ± 0.1^{b}	0.1 ± 0.0^{b}	0.4 ± 0.1^{a}	0.3 ± 0.1^{a}	0.1 ± 0.0^{b}
22:5n-6	0.9 ± 0.1^{b,c}	1.3 ± 0.1^{a}	1.2 ± 0.1^{a}	1.1 ± 0.2^{a,b}	0.7 ± 0.1^{c}
Total n-6 PUFA	5.2 ± 0.4^{c}	6.9 ± 0.3^{c}	28.2 ± 1.7^{a}	24.8 ± 1.9^{b}	8.1 ± 0.0^{c}
18:3n-3	0.2 ± 0.1^{a}	0.3 ± 0.1^{a}	n.d.^{c}	0.1 ± 0.0^{b}	n.d.^{c}
20:3n-3	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	n.d.^{b}	n.d.^{b}	n.d.^{b}
20:4n-3	0.7 ± 0.1^{a}	0.5 ± 0.1^{b}	0.1 ± 0.0^{d}	0.1 ± 0.0^{d}	0.2 ± 0.1^{c}
20:5n-3	5.0 ± 0.1^{a}	5.9 ± 0.5^{a}	3.2 ± 0.1^{b}	3.0 ± 0.6^{b}	6.1 ± 0.9^{a}
22:5n-3	2.2 ± 0.3^{a}	1.9 ± 0.1^{a}	1.3 ± 0.1^{b}	1.2 ± 0.2^{b}	1.4 ± 0.1^{b}
22:6n-3	41.5 ± 0.7^{a,b}	42.7 ± 1.2^{a}	36.0 ± 0.4^{c}	36.0 ± 1.1^{c}	39.1 ± 1.1^{b}
Total n-3 PUFA	49.8 ± 0.7^{a}	51.5 ± 0.8^{a}	40.6 ± 0.3^{c}	40.4 ± 1.3^{c}	46.8 ± 0.3^{b}
Total PUFA	56.9 ± 0.8^{d}	60.2 ± 1.0^{c}	69.6 ± 1.5^{a}	66.1 ± 0.9^{b}	55.8 ± 0.3^{d}
n-3/n-6	9.7 ± 0.7^{a}	7.5 ± 0.2^{b}	1.4 ± 0.1^{d}	1.6 ± 0.2^{d}	5.9 ± 0.1^{c}

[a]See Table 3 footnote. See Table 2 for company sources.

TABLE 6
Effects of Diet on the Fatty Acid Compositions of Phosphatidylserine from the Liver of Atlantic Salmon[a]

	Diet				
Fatty acid	Fosol	Tuna oil/Marinol	Sunflower oil	Borage oil	Olive oil
Total saturates	28.4 ± 1.5[b]	32.1 ± 0.8[a,b]	31.0 ± 0.2[a,b]	32.4 ± 2.6[a]	28.4 ± 0.8[b]
Total monoenes	19.1 ± 1.6[a]	11.6 ± 1.1[b]	8.8 ± 0.4[b]	9.4 ± 1.5[b]	19.0 ± 2.3[a]
18:2n-6	1.5 ± 0.1[c]	1.1 ± 0.1[c]	11.0 ± 1.3[a]	5.2 ± 2.0[b]	2.4 ± 0.5[b,c]
18:3n-6	n.d.[b]	n.d.[b]	0.1 ± 0.0[b]	0.3 ± 0.1[a]	0.3 ± 0.1[a]
20:2n-6	0.8 ± 0.1[b]	0.5 ± 0.2[b]	3.3 ± 0.4[a]	0.9 ± 0.4[b]	1.1 ± 0.7[b]
20:3n-6	0.1 ± 0.1[c]	0.1 ± 0.1[c]	2.5 ± 0.3[a]	3.6 ± 0.9[a]	0.8 ± 0.0[b]
20:4n-6	0.6 ± 0.3[b]	1.0 ± 0.4[a,b]	1.7 ± 0.4[a]	1.6 ± 0.4[a]	0.4 ± 0.0[b]
22:4n-6	0.1 ± 0.0[c]	0.1 ± 0.1[b,c]	0.4 ± 0.2[a]	0.3 ± 0.1[a,b]	trace[c]
22:5n-6	0.8 ± 0.0[b,c]	1.4 ± 0.1[a]	0.8 ± 0.2[b,c]	1.0 ± 0.2[b]	0.6 ± 0.1[c]
Total n-6 PUFA	3.9 ± 0.4[c]	4.2 ± 0.3[c]	19.8 ± 1.1[a]	12.9 ± 3.5[b]	5.6 ± 1.0[c]
18:3n-3	0.5 ± 0.2[a]	0.7 ± 0.1[a]	n.d.[b]	0.2 ± 0.2[b]	0.2 ± 0.0[b]
20:3n-3	0.2 ± 0.0[a]	0.1 ± 0.0[b]	n.d.[c]	n.d.[c]	n.d.[c]
20:4n-3	0.5 ± 0.0[a]	0.3 ± 0.1[b]	0.1 ± 0.1[c]	trace[c]	0.2 ± 0.0[b]
20:5n-3	1.3 ± 0.2[a]	1.3 ± 0.2[a]	1.0 ± 0.1[a,b]	0.7 ± 0.2[b]	1.2 ± 0.1[a]
22:5n-3	1.9 ± 0.3	1.9 ± 0.2	1.3 ± 0.2	1.5 ± 0.4	1.4 ± 0.2
22:6n-3	38.9 ± 1.3[a,b]	43.4 ± 1.8[a]	33.2 ± 1.2[b]	38.2 ± 2.4[a,b]	35.6 ± 3.4[b]
Total n-3 PUFA	43.3 ± 1.6[a,b]	47.7 ± 1.6[a]	35.6 ± 1.3[c]	40.6 ± 2.5[b,c]	38.7 ± 3.6[b,c]
Total PUFA	49.2 ± 1.6[b,c]	53.0 ± 1.7[a,b]	56.2 ± 0.4[a]	54.2 ± 1.2[a]	45.3 ± 2.6[c]
n-3/n-6	11.3 ± 1.0[a]	11.5 ± 0.6[a]	1.8 ± 0.2[c]	3.3 ± 1.0[c]	7.2 ± 2.0[b]

[a]See Table 3 footnote; trace value, < 0.05%. See Table 2 for company sources.

desaturation of [1-^{14}C]18:2n-6 was much less affected by diet but, as with the desaturation of [1-^{14}C]18:3n-3, the fish fed BO displayed significantly greater recoveries of radioactivity in Δ6- (over 2.4-fold greater than any other diet), Δ5-, and total-desaturation products (Table 9). There was no significant difference between the two diets containing fish oil, FO and MO/TO, in the desaturation of either [1-^{14}C]18:3n-3 or [1-^{14}C]18:2n-6 although the MO/TO diet consistently gave the lowest percentages of radioactivity recovered in almost all the fatty acid fractions.

TABLE 7
Effects of Diet on the Fatty Acid Compositions of Phosphatidylinositol from the Liver of Atlantic Salmon[a]

	Diet				
Fatty acid	Fosol	Tuna oil/Marinol	Sunflower oil	Borage oil	Olive oil
Total saturates	34.9 ± 1.2[b]	35.3 ± 1.9[a,b]	38.1 ± 0.7[a]	36.9 ± 1.0[a,b]	30.0 ± 0.7[c]
Total monoenes	9.3 ± 1.1[b]	8.1 ± 1.6[b,c]	4.8 ± 0.7[d]	6.2 ± 0.7[c,d]	13.7 ± 0.4[a]
18:2n-6	0.4 ± 0.1[b]	0.4 ± 0.1[b]	2.3 ± 0.5[a]	2.3 ± 0.6[a]	1.4 ± 0.1[c]
18:3n-6	0.6 ± 0.0[a,b]	0.8 ± 0.2[a]	0.1 ± 0.0[c]	0.3 ± 0.3[b,c]	0.2 ± 0.0[c]
20:2n-6	0.2 ± 0.0[b]	0.1 ± 0.1[b]	0.7 ± 0.1[a]	0.3 ± 0.1[b]	0.6 ± 0.2[a]
20:3n-6	0.7 ± 0.1[c]	0.6 ± 0.2[c]	4.2 ± 0.6[b]	5.9 ± 1.1[a]	6.9 ± 0.7[a]
20:4n-6	26.0 ± 1.4[a]	29.7 ± 1.7[a]	31.2 ± 1.0[a]	26.3 ± 4.3[a]	18.6 ± 0.7[b]
22:4n-6	0.1 ± 0.1	trace	0.1 ± 0.1	0.1 ± 0.1	trace
22:5n-6	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
Total n-6 PUFA	28.7 ± 1.2[c]	32.0 ± 1.5[b,c]	38.8 ± 1.7[a]	35.5 ± 2.4[a,b]	27.9 ± 0.3[c]
18:3n-3	n.d.	trace	n.d.	0.2 ± 0.1	0.1 ± 0.1
20:4n-3	0.3 ± 0.1[a]	0.3 ± 0.1[a]	trace[b]	trace[b]	0.3 ± 0.1[a]
20:5n-3	3.1 ± 0.8[a]	2.4 ± 0.5[a,b]	1.2 ± 0.1[b,c]	0.9 ± 0.4[c]	3.6 ± 0.2[a]
22:5n-3	1.9 ± 0.1[a]	1.6 ± 0.0[b]	0.9 ± 0.1[d]	1.1 ± 0.1[c,d]	1.4 ± 0.2[b,c]
22:6n-3	18.9 ± 1.4[a]	17.5 ± 0.9[a]	13.3 ± 1.0[b]	16.0 ± 1.4[a,b]	17.6 ± 0.5[a]
Total n-3 PUFA	24.2 ± 1.1[a]	21.8 ± 0.8[a]	15.4 ± 1.2[b]	18.2 ± 1.6[b]	23.0 ± 0.8[a]
Total PUFA	53.1 ± 0.0[a,b]	54.0 ± 0.6[a]	54.3 ± 1.4[a]	53.8 ± 0.9[a,b]	52.0 ± 0.2[b]
n-3/n-6	0.9 ± 0.1[a]	0.7 ± 0.1[b,c]	0.4 ± 0.0[d]	0.5 ± 0.1[c,d]	0.8 ± 0.1[a,b]

[a]See Table 3 footnote. See Table 2 for company sources.

TABLE 8
Effects of Diet on the Recovery of Radioactivity Unmetabolized or in Products Only Elongated by Isolated Hepatocytes from Atlantic Salmon (*Salmo salar*) Incubated with [1-^{14}C]18:3n-3 or [1-^{14}C]18:2n-6[a]

Fatty acid fraction	Diet				
	Fosol	Tuna oil/Marinol	Sunflower oil	Borage oil	Olive oil
[1-^{14}C]18:3n-3					
18:3n-3	67.9 ± 2.8^{a}	72.4 ± 1.6^{a}	65.8 ± 2.1^{a}	54.3 ± 2.2^{b}	65.4 ± 3.3^{a}
20:3n-3	19.5 ± 0.9^{a}	18.7 ± 1.9^{a}	13.4 ± 0.9^{a}	5.2 ± 1.2^{b}	13.3 ± 1.5^{a}
[1-^{14}C]18:2n-6					
18:2n-6	71.1 ± 1.0^{a}	73.1 ± 1.0^{a}	75.1 ± 2.8^{a}	66.8 ± 1.6^{b}	76.4 ± 1.3^{a}
20:2n-6	21.6 ± 0.6^{a}	21.3 ± 1.6^{a}	15.4 ± 3.5^{a,b,c}	13.9 ± 0.8^{c}	14.2 ± 0.6^{b,c}

[a]Hepatocytes were isolated and incubated with either [1-^{14}C]18:3n-3 or [1-^{14}C]18:2n-6 as described in the Materials and Methods section. Results are expressed as the amount of radioactivity recovered in the fractions as percentages of total radioactivity recovered. The significance of differences between the dietary treatments was analyzed by one-way analysis of variance (ANOVA). Mean values with different superscript letters within a given row are significantly different ($P < 0.05$) as determined by Tukey's multiple range test. The results of two-way ANOVA were: substrate—significant for both the C_{18} ($P = 0.0000$; mean values for 18:3 and 18:2 being 65.9 and 72.8) and C_{20} ($P = 0.0000$; mean values for 20:3 and 20:2 being 14.3 and 17.4); diet—significant for both the C_{18} [$P = 0.0000$; mean value for borage oil (BO) being significantly lower than all other diets at 60.7, with other diets varying between 69.6 and 72.8 and no significant difference between them] and C_{20} ($P = 0.0019$; mean value for BO being significantly lower than all other diets at 9.5, with other diets varying between 13.7 and 20.5 with some significant differences). See Table 2 for company sources.

TABLE 9
Effects of Diet on the Desaturation of [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6 by Isolated Hepatocytes from Atlantic Salmon (*Salmo salar*)[a]

Desaturation products	Diet				
	Fosol	Tuna oil/Marinol	Sunflower oil	Borage oil	Olive oil
[1-^{14}C]18:3n-3					
Δ6	7.5 ± 0.6^{b}	5.9 ± 0.5^{b}	11.7 ± 0.9^{a}	13.4 ± 0.9^{a}	10.8 ± 0.8^{a}
Δ5	4.8 ± 2.4^{b,c}	2.4 ± 0.5^{c}	7.2 ± 1.8^{b}	22.9 ± 0.9^{a}	8.8 ± 1.0^{b}
Δ6*	0.3 ± 0.1^{c}	0.6 ± 0.1^{c}	1.9 ± 0.5^{b}	4.2 ± 0.8^{a}	1.7 ± 0.0^{b,c}
Total	12.6 ± 1.9^{c}	8.9 ± 0.3^{c}	20.8 ± 2.5^{b}	40.5 ± 1.1^{a}	21.3 ± 1.8^{b}
[1-^{14}C]18:2n-6					
Δ6	4.5 ± 2.0^{b}	3.3 ± 1.4^{b}	6.3 ± 0.6^{b}	15.4 ± 0.5^{a}	6.2 ± 0.9^{b}
Δ5	1.4 ± 0.1^{b}	0.8 ± 0.2^{b}	1.3 ± 0.3^{b}	2.4 ± 0.1^{a}	1.0 ± 0.0^{b}
Δ6*	1.4 ± 0.2	1.5 ± 0.3	1.9 ± 0.4	1.5 ± 0.1	2.2 ± 0.2
Total	7.3 ± 1.6^{b,c}	5.6 ± 1.8^{c}	9.5 ± 0.8^{b}	19.3 ± 0.8^{a}	9.4 ± 0.7^{b,c}

[a]Hepatocytes were isolated and incubated with either [1-^{14}C]18:3n-3 or [1-^{14}C]18:2n-6 as described in the Materials and Methods section. Results are expressed as the amount of radioactivity recovered in the various desaturated products as percentages of total radioactivity recovered. The significance of differences between the dietary treatments was analyzed by one-way analysis of variance (ANOVA). Mean values with different superscript letters within a given row are significantly different ($P < 0.05$) as determined by Tukey's multiple range test. Δ6, products of Δ6 desaturation (18:4 and 20:4 for n-3, and 18:3 and 20:3 for n-6); Δ5, products of Δ5 desaturation (20:5 and 22:5 for n-3, and 20:4 and 22:4 for n-6); Δ6*, hexaene products for n-3 and pentaene products for n-6. The results of two-way ANOVA were: diet—Δ6, Δ5, Δ6* and total all significant ($P = 0.0000$); substrate—Δ6, Δ5 and total all significant ($P = 0.0000$), but Δ6* ns, $P = 0.7342$ with the differences (Tukey's) being Δ6, n-3 > n-6 (9.66 vs. 6.74); Δ5, n-3 > n-6 (8.48 vs. 1.33); total, n-3 > n-6 (19.76 vs. 9.75); interaction—Δ6, Δ5, Δ6* and total all significant $P = 0.0000$ except Δ6 $P = 0.0006$. See Table 2 for company sources.

DISCUSSION

In the present study, the use of intact hepatocytes to determine the metabolism of fatty acids by desaturation and elongation was preferred to the use of microsomes. Therefore, it should be noted that the present data do not measure desaturase enzyme activity as directly as with microsomal preparations. Microsomal preparations, though, are limited, particularly in relation to 18:3n-3 metabolism in fish, in that the production of DHA cannot be determined in microsomes alone because peroxisomes are also required (7). The utilization of intact hepatocytes enables the whole desaturation/elongation pathway to be assayed. Furthermore, dietary influences of membrane fatty acid composition will extend to all cell membranes including plasma membranes. Therefore, altered membrane fatty acid compositions may affect other membrane processes such as fatty acid uptake into the cell (43). Fatty acids also have to be transported intracellularly and activated by acyl-CoA synthetase, and so there are other enzyme activities whose modulation could contribute to the overall level of fatty acyl desaturation observed (44). Nonetheless, assaying fatty acyl desaturation/elongation in intact hepatocytes can

arguably be regarded as more accurately reflecting the effect of diet on all these processes compared to subcellular microsomal preparations. However, diet is known to directly affect desaturase activities in mammals (5,19) and the differential effects of an individual diet on Δ6, Δ5 and Δ6* activities suggest that much of the dietary effect may be on the desaturation/elongation enzyme systems themselves.

The results clearly demonstrated that 18:3n-3 and n-3 PUFA in general were the preferred substrates for desaturation in salmon hepatocytes, irrespective of diet. Less [1-^{14}C]18:3n-3 was recovered unmetabolized with a greater proportion recovered as desaturated products, compared to [1-^{14}C]18:2n-6. This observation is in agreement with previous studies in rats (3), rainbow trout (45), Atlantic salmon parr (23), and established fish cell lines (46). In contrast, it was noteworthy that a greater proportion of [1-^{14}C]18:2n-6 was converted by elongation to 20:2n-6 compared to the production of 20:3n-3 from [1-^{14}C]18:3n-3.

The MO/TO diet was formulated to contain more n-3 PUFA than the FO diet, with less of the long-chain monoenes, such as 20:1 and 22:1, characteristic of Northern Hemisphere fish oil. However, it was clear from the liver lipid data that the MO/TO diet resulted in fatty acid compositions very similar to the FO diet, and this was reflected in the fatty acid metabolism data where there were no differences between the two diets containing fish oil. However, the vegetable oil diets, SO, BO and OO, resulted in very significant effects on the liver phospholipid fatty acid compositions compared to the fish oil diets, and there were also clear differences between the diets containing fish oil and vegetable oils in their effects on PUFA metabolism. Most obvious was the fact that the desaturation of [1-^{14}C]18:3n-3 was considerably greater in hepatocytes from fish fed diets containing vegetable oils. The desaturation of [1-^{14}C]18:2n-6 was less affected but also tended to be increased in fish fed diets containing vegetable oils. It is probably appropriate to interpret such data as inhibition of fatty acyl desaturation by long-chain n-3 PUFA in the fish oil diets since such long-chain n-3 PUFA are known to be inhibitory to the desaturation of both 18:3n-3 and 18:2n-6 in rats (13,47,48) and rainbow trout (45). The present study confirms that in this respect fish are no different from mammals and that diets containing long-chain n-3 PUFA-rich fish oils suppress hepatic fatty acyl desaturation. Similarly, it was noteworthy that there was very little difference between SO and OO in their effects on hepatic fatty acyl desaturation in salmon. Previously it was shown that there was no difference in Δ6-desaturase activity in duodenal mucosa microsomes from dogs fed SO or OO (49).

Although the difference between diets containing fish oil and diets containing vegetable oil was very clear, the most significant finding in the present study was that BO greatly increased hepatic fatty acid desaturation. In hepatocytes from fish fed BO, the total desaturation products were double that found with any other diet and almost fourfold that of the lowest level of desaturation observed in the MO/TO diet. Dietary 18:3n-6 has been reported to have variable and conflicting effects in rats. An early study reported that 18:3n-6 in the form of evening primrose oil did not modify liver Δ6- or Δ5-desaturase activities in rats after 11 wk of feeding (50). However, in young rats fed a diet containing 18:3n-6, Δ5-desaturase activity was depressed (51). In contrast, a diet containing evening primrose oil depressed liver Δ6-desaturase activity in 8-mon-old rats but not in 3-wk-old rats (52). Borage oil (18:3n-6 only) and blackcurrant oil (18:3n-6 and 18:4n-3) both tended to decrease liver microsomal Δ6- and Δ5-desaturase activities in younger rats but tended to increase both activities in older rats (53). Clearly, age of the animal and possibly the different fatty acid compositions of the oils containing 18:3n-6 that were used in these studies have contributed to the conflicting data obtained. Certainly, rat liver Δ6-desaturase activity decreases with age (5), and it was shown that dietary supplementation of GLA in the form of evening primrose oil could reverse this aging effect in rats (54). There are no data on the effects of aging on desaturase activities in fish, but considering the very significant results from the present study, it appears that hepatic fatty acyl desaturation fish may be more affected by dietary GLA than in mammals.

The present study also revealed some other dietary effects that were noteworthy. Two-way ANOVA showed significant interactions indicating that the effects of the diets were dependent upon the substrate fatty acid used. For instance, the greatest effect of BO on the desaturation of 18:3n-3 was at the Δ5 level, whereas it was at the Δ6 level for the desaturation of 18:2n-6. Most previous work in mammals has focused on the effects of diet on Δ6-desaturase activity, regarded as the rate-limiting step of the desaturase pathway (5,19). Although some studies have also measured Δ5 desaturation, few have investigated the effects on 18:3n-3 metabolism or of dietary 18:3n-6 (12).

As the diet containing BO was significantly different from the diets containing SO and OO in its effects on fatty acid desaturation, the question must be what were the major differences in liver fatty acid compositions between the diets containing vegetable oil that may be influencing factors. Liver total lipid of salmon fed the BO, SO, and OO diets all had very similar levels of EPA, DHA, and total n-3 PUFA, and although there were slight differences in the distribution of n-3 PUFA in individual phospholipids between the diets containing vegetable oil, it was not likely that differences in n-3 PUFA inhibition were responsible. Total PUFA levels in liver phospholipids in the BO-fed fish were also very high indicating that a generally low PUFA level was not a factor. Similarly, the levels of 18:2n-6 and 18:3n-3 did not correlate with levels of desaturation activity as the level of 18:2n-6 in BO-fed fish was intermediate between SO and OO, and hepatic 18:3n-3 levels were very low in all diets. However, 20:3n-6, and to a lesser extent AA, were generally significantly higher in liver lipids in the BO-fed fish compared to all the other diets. AA is the preferred precursor substrate PUFA for eicosanoid synthesis in fish, despite EPA being more abundant in tissue phospholipids (15,55), and increasing the levels of 20:3n-6 in cell phospholipids significantly increases the

production of 1-series prostaglandins and inhibits the production of 2- and 3-series prostaglandins in fish (55). There are few data on the effects of eicosanoids on fatty acid desaturation, but it is possible that modulation of eicosanoids may play a role in the effect of GLA on hepatic fatty acid desaturation in the present study.

This was not a nutritional study, and so the direct application of the results in the modification of salmon diets in the future is limited. However, in the present study the vegetable oil diets did not result in significantly lower final weights compared to the control FO diet. There was considerable variation though, and fish on the SO diet were significantly lighter than fish on the TO/MO diet, suggesting that diets with very high 18:2n-6 were potentially detrimental. The study also showed that the vegetable oil diets increased the desaturation of [1-^{14}C]18:3n-3 in salmon liver, consistent with other studies that have shown that salmon can utilize vegetable oils, providing the oils contain sufficient 18:3n-3 to satisfy essential fatty acid requirements (28,30–32). In the previous studies, linseed oil diets were not associated with adverse effects, whereas sunflower oil diets were shown to have potentially detrimental effects in salmon (31). Recently, however, we have shown that a blend of 18:3n-3 and 18:2n-6 fed to salmon parr enhanced hepatic desaturase activities and were beneficial in effecting successful seawater transfer (23). Similarly, the present study has indicated that if vegetable oils are used to replace fish oils in salmon smolt diets, the inclusion of borage oil in the blends may be beneficial.

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Docosahexaenoic Acid-Rich Fish Oil Does Not Enhance the Elevation of Serum Transaminase and Liver Triacylglycerol Induced by Carbon Tetrachloride in Mice

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ABSTRACT: Carbon tetrachloride (CCl_4) is metabolized to trichloromethyl radical and induces liver injury with elevated serum transaminase and increased hepatic triacylglycerols (TG). To answer the question whether dietary polyunsaturated fatty acids (PUFA) enhance free radical-mediated liver injury, a docosahexaenoic acid (DHA)-rich fish oil (FO) or a saturated and monounsaturated fatty acid-rich beef tallow diet was fed to mice for 4 wk and then CCl_4 was administered. When thiobarbituric acid-reactive substances (TBARS) were measured in the absence of antioxidant, the FO diet and CCl_4 treatment markedly increased liver TBARS values synergistically, apparently supporting the interpretation that the highly autoxidizable DHA accelerates lipid peroxidation induced by CCl_4. However, no such marked interaction was observed between diet and CCl_4 treatment in liver TBARS values measured in the presence of an antioxidant in the assay mixtures as well as in conjugated diene contents. Furthermore, the FO diet did not enhance CCl_4-induced elevation of serum transaminase but lowered liver TG levels. The proportion of DHA, the most easily autoxidizable among common PUFA, was increased but those of eicosanoid precursors were decreased in liver phospholipids by CCl_4 treatment, possibly reflecting the inflammation-related mobilization of eicosanoid precursors but not lipid peroxidation. These results indicate that dietary enrichment with DHA does not enhance CCl_4-induced liver injury through the so-called free radical-mediated propagative autoxidation of DHA in mice.

Lipids 32, 1249–1255 (1997).

Dietary fish oil (FO) rich in the n-3 series fatty acids eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) has been suggested to be beneficial for cardiovascular and inflammatory diseases (1,2). Inhibition of eicosanoid synthesis from arachidonic acid (20:4n-6, AA) by n-3 polyunsaturated fatty acids (PUFA) as well as their hypolipidemic effects is assumed to be possibly responsible for their beneficial effects (3). On the other hand, these n-3 PUFA have more methylenic hydrogens between the two double bonds than does arachidonic acid (AA), and in air are more easily autoxidized to lipid peroxides (4). In fact, liver microsomal membranes from rats fed an FO diet have been shown to be more susceptible to lipid peroxidation *in vitro* (5). In addition, elevated levels of lipid peroxides and reduced concentrations of α-tocopherol, an endogenous antioxidant, in blood and tissues have been observed in rats fed FO diets (6), although another study reported that the elevation of lipid peroxides caused by an FO diet was not prevented completely even in the presence of added antioxidants (7). These observations led to the concept that an intake of excess amounts of n-3 PUFA over n-6 PUFA stimulates so-called free radical-mediated diseases by enhancing lipid peroxidation in tissues, impairing the α-tocopherol status. A similar concept has been accepted widely as a free radical/lipid peroxide theory of aging, carcinogenesis, and atherosclerosis (8–12).

Carbon tetrachloride (CCl_4) has been used extensively to induce liver injury in experimental animals. CCl_4 is metabolized by liver microsomal cytochrome P-450 to the trichloromethyl radical ($CCl_3\cdot$), which incorporates O_2 to form the trichloromethylperoxy radical ($CCl_3O_2\cdot$) which then withdraws allylic hydrogens from PUFA to initiate lipid peroxidation (13). The peroxidative degradation of membrane PUFA is implicated in liver cell damage (14). Furthermore, the administration of α-tocopherol has been reported to lower the elevated lipid peroxide level and protect against liver injury in rats treated with CCl_4 (15–17).

This study was initially designed to assess the possibility that dietary supplementation with highly autoxidizable DHA may cause adverse effects on CCl_4-induced liver injury in the presence of α-tocopherol.

MATERIALS AND METHODS

Animals and diets. Female ICR mice at 3 wk of age (Shizuoka Laboratory Animals Co., Ltd., Shizuoka, Japan) were fed the test diets for 4 wk. The test diets were prepared by mixing a conventional diet (CE-2; Clea Japan, Inc., Tokyo, Japan) con-

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Abbreviations: AA, arachidonic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase, BHT, butylated hydroxytoluene; BT, beef tallow; CCl_4, carbon tetrachloride; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; PI, peroxidizability index; PL, phospholipid; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid-reactive substances; TG, triacylglycerol.

taining 3% (w/w) lipids and beef tallow (Wako Pure Chemical Co., Osaka, Japan) or DHA-rich FO (Harima Chemicals Inc., Tokyo, Japan) at 9:1 weight ratio and the final lipid content was 12.7% (w/w) (29.7 energy percentage). The fatty acid compositions of the diets are shown in Table 1. A peroxidizability index (PI) was calculated by Equation 1 (Ref. 18):

$$\% \text{ monoenoate} \times 0.025 + \% \text{ dienoate} \times 1 + \% \text{ trienoate} \times 2 + \% \text{ tetraenoate} \times 4 + \% \text{ pentaenoate} \times 6 + \% \text{ hexaenoate} \times 8 \quad [1]$$

α- and γ-Tocopherol concentrations in the test diets were adjusted to 190 and 530 mg/kg of diet, respectively, by adding α-tocopherol (Sigma, St. Louis, MO) and γ-tocopherol (Ohta Oil Co., Ltd., Aichi, Japan). The test diets were kept frozen at −20°C for less than 2 wk until serving. Diets were replaced every other day to keep the peroxide values of ingested diets below 30 meq/kg of oil. Mice were kept at 23 ± 2°C and given free access to the test diets.

CCl_4 administration. After 4 wk of feeding, mice were injected subcutaneously with 1 mL of CCl_4/kg dissolved in olive oil or with olive oil only (vehicle-treated control). Mice were sacrificed 24 h after the injection to obtain blood and liver, or at 30 min after the injection for the determination of conjugated dienes (19). The excised livers were frozen quickly in liquid nitrogen. Sera and livers were stored at −20 and −80°C, respectively, until analysis.

Determination of thiobarbituric acid-reactive substances (TBARS) in liver. Liver was weighed and homogenized in cold 1.15% KCl to prepare 10% (wt/vol) homogenates. The content of TBARS in the liver homogenate was measured according to the method of Ohkawa *et al*. (20) or Kikugawa *et al*. (21). The two methods differed in that the coloring reactions were developed in the absence (20) or the presence (21) of butylated hydroxytoluene (BHT) (200 μg/mL of reaction mixture). A calibration curve for the quantitation of liver TBARS content was established by using known amounts of malonaldehyde-bis(dimethylacetal) (Aldrich, Milwaukee, WI).

Determination of conjugated dienes in liver. Liver was weighed and homogenized in cold 0.3 M sucrose and 3 mM EDTA (pH 7.4) to yield 10% (wt/vol) homogenates. The conjugated diene content of total lipids extracted by Bligh and Dyer method (22) was determined by measuring the optical density at 233 nm and was expressed as nmol of diene equivalent/gram liver ($\varepsilon = 27{,}000\ M^{-1}\ cm^{-1}$) (23).

Determination of alanine aminotransferase and aspartate aminotransferase activities in serum. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using a spectrophotometric method (24). For the determination of serum ALT activity, serum samples were incubated in 500 mM L-alanine, 12 mM 2-oxoglutarate, 100 mM Tris, 0.18 mM NADH, 0.11 mM pyridoxal phosphate, and 1200 U/L lactate dehydrogenase (pH 7.3). On the other hand, serum samples were incubated in 240 mM L-aspartate, 12 mM 2-oxoglutarate, 91 mM Tris, 0.16 mM NADH, 0.11 mM pyridoxal phosphate, 420 U/L lactate dehydrogenase, and 930 U/L malate dehydrogenase (pH 7.8) for AST determination.

Determination of tocopherols. α- and γ-Tocopherols were extracted from serum with *n*-hexane and those from liver and diet were extracted with chloroform/methanol (22). The extract of diets was saponified with 1 N ethanolic KOH in the presence of 5.5% (wt/vol) of pyrogallol and, the tocopherols were extracted with *n*-hexane. The tocopherol isomers were measured by straight-phase high-performance liquid chromatography (25,26) on a LiChrosorb 5 NH_2 column (4.6 × 150 mm) (GL Sciences Inc., Tokyo, Japan) equilibrated with *n*-hexane/isopropanol (98:2, vol/vol) (0.8 mL/min) at 40°C. The elution of tocopherol isomers was monitored fluorometrically (excitation at 298 nm and emission at 325 nm). 2,2,5,7,8-Pentamethyl-6-hydroxychroman (Wako) was added prior to the extraction steps as an internal standard.

Analyses of triacylglycerol (TG) and phospholipid (PL) in serum and liver. Total lipids were extracted from serum and liver according to the method of Bligh and Dyer (22). TG and PL were separated by thin-layer chromatography on a silica gel plate with petroleum ether/diethyl ether/acetic acid (80:30:1, by vol) as a developing solvent. The lipids were visualized under ultraviolet light after spraying with an ethanolic solution of Rhodamine 6G (Tokyo Kasei; Tokyo, Japan) and were extracted from the adsorbent by the method of Bligh and Dyer (22). Fatty acids in the TG and PL fractions were converted to methyl esters by treatment with 5% (wt/vol) HCl in methanol for 1 h in a boiling water bath and then quantitated by gas–liquid chromatography using a capillary column (DB-225; J&W Scientific, Folsom, CA). A triheptadecanoylglycerol and a 1,2-diheptadecanoyl-L-α-phosphatidylcholine

TABLE 1
Fatty Acid Composition of Experimental Diets[a]

Fatty acid	Beef tallow diet	Fish oil diet
14:0	2.0	2.0
16:0	23.5	14.7
16:1	2.2	4.5
18:0	13.6	2.5
18:1n-9[b]	39.3	16.2
18:2n-6	15.8	16.7
18:3n-3	1.2	1.8
20:1	0.6	1.3
20:4n-6	0.1	1.7
20:5n-3	0.6	7.0
22:5n-6	n.d.	1.6
22:6n-3	0.6	29.5
n-6/n-3[c]	6.43	0.53
PI[d]	30.0	316.5

[a]The fatty acid composition in the diets (% of total fatty acids) was analyzed by gas–liquid chromatography. n.d., not detected.
[b]The position of the double bond numbered from the methyl terminus is designated as n-9, n-6, or n-3.
[c]n-6/n-3 indicates the total n-6 fatty acids/total n-3 fatty acids ratio.
[d]Peroxidizability index = (% monoenoate × 0.025) + (% dienoate × 1) + (% trienoate × 2) + (% tetraenoate × 4) + (% pentaenoate × 6) + (% hexaenoate × 8) (Ref. 18).

(Sigma) were added prior to lipid extraction as internal standards for the quantitation of TG and PL, respectively.

Statistical analysis. Data are presented as means ± SD. The significance of the effects of diet, CCl_4 treatment, and their interaction were analyzed by a two-way analysis of variance (StatView-J 4.02, Abacus Concepts Inc., Berkeley, CA). A probability below 0.05 was considered significant.

RESULTS

Content of TBARS in liver. Determinations of TBARS in tissues have been used extensively as an assay of lipid peroxides. We first performed the TBARS assay without adding antioxidant to the reaction mixture for the coloring reaction, which corresponds to the original method reported by Ohkawa *et al.* (20). Liver TBARS contents were significantly increased by each FO diet ($P < 0.0001$) or CCl_4 treatment ($P < 0.0001$), and their interaction was also significant ($P < 0.0001$), indicating that an FO diet and CCl_4 treatment synergistically increased TBARS contents of liver (Fig. 1A). In contrast, when the TBARS assay was performed in the presence of BHT in the reaction mixture for the coloring reaction (21), the overall liver TBARS contents decreased to approximately one-tenth of those observed without BHT (Fig. 1B). The TBARS content was significantly higher in the FO diet group than in the beef tallow (BT) diet group ($P < 0.05$), but the significant effect of CCl_4 treatment observed in the original TBARS assay (Fig. 1A) disappeared. We confirmed that BHT itself did not interfere directly with the coloring reaction of thiobarbituric acid and malondialdehyde, indicating that most of the TBARS in the absence of BHT were actually produced during the assay.

Conjugated diene content in liver. Since Kim and LaBella (19) have reported that the levels of conjugated dienes in liver peaks 30 min after CCl_4 administration, and then decreases to near the unstimulated levels by 24 h, we determined conjugated diene contents in liver at 30 min after CCl_4 treatment (Fig. 2). The conjugated diene contents of liver were slightly but significantly higher in the FO diet group than in the BT diet group ($P < 0.05$) and tended to increase after CCl_4 treatment ($0.05 < P < 0.1$), although no significant interaction between the diet and CCl_4 treatment was observed.

Serum and liver α-tocopherol concentrations. As α-tocopherol status might be further impaired when the FO diet was combined with CCl_4 treatment (6,27), we determined α-tocopherol concentrations in the sera and livers of mice fed BT or FO diets in the absence or presence of CCl_4 treatment (Fig. 3). In the vehicle-treated mice, the serum α-tocopherol concentration in the FO diet group was significantly lower than in the BT diet group ($P < 0.01$) (Fig. 3A). CCl_4 treatment significantly decreased the serum α-tocopherol concentration in the BT diet group but not in the FO diet group. On the other hand, liver α-tocopherol concentrations were significantly lower in the FO diet group than in the BT diet group ($P < 0.0001$) but were not influenced by CCl_4 treatment (Fig. 3B).

CCl_4-induced liver injury. As shown above, the FO diet and CCl_4 treatment synergistically increased the TBARS content in liver. Therefore, we examined the severity of liver injury by measuring serum transaminase activities, ALT and AST, after CCl_4 treatment. CCl_4 treatment markedly increased these transaminase activities in serum in both the BT and FO diet groups as compared with the respective vehicle-treated control groups (Fig. 4). However, no significant difference was observed in both ALT and AST activities of the two dietary groups.

TG and PL in serum and liver. Feeding the FO diet lowered the serum PL concentration significantly ($P < 0.01$) (707 ± 129 mg/mL in the BT diet group and 464 ± 112 mg/mL in the FO diet group, $n = 10$), but serum TG concentrations were not much different ($0.05 < P < 0.1$) (751 ± 340

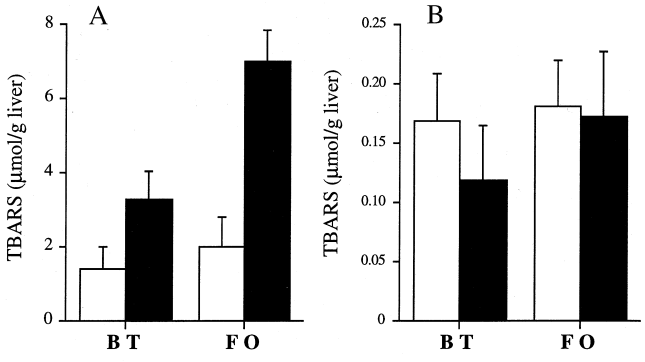

FIG. 1. Liver thiobarbituric acid-reactive substances (TBARS) contents determined without (A) or with (B) butylated hydroxytoluene (BHT) in the reaction mixture (200 μg/mL). Female ICR mice were fed beef tallow (BT) or fish oil (FO) diets for 4 wk and were sacrificed 24 h after vehicle (open bar) or carbon tetrachloride (CCl_4) (solid bar) treatments (1 mL of CCl_4/kg). In the two-way analysis of variance (ANOVA), there were effects of diet ($P < 0.0001$), CCl_4 treatment ($P < 0.0001$) and the interaction between diet and CCl_4 treatment ($P < 0.0001$) in liver TBARS (−BHT) (A); and there was an effect of diet ($P < 0.05$) in liver TBARS (+BHT) (B). Each column with a bar represents the mean ± SD for eight mice.

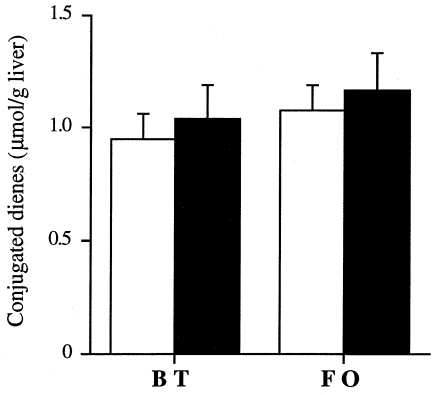

FIG. 2. Liver conjugated diene contents in mice fed BT or FO diets for 4 wk. Samples were taken 30 min after vehicle (open bar) or CCl_4 (solid bar) treatments. Liver conjugated dienes were assayed by measuring optical density at 233 nm and expressed as nmol of diene equivalent/gram liver. In the two-way ANOVA, there was an effect of diet ($P < 0.05$) but not CCl_4 treatment ($P = 0.082$). Each column with a bar represents the mean ± SD for seven to eight mice. See Figure 1 for abbreviations.

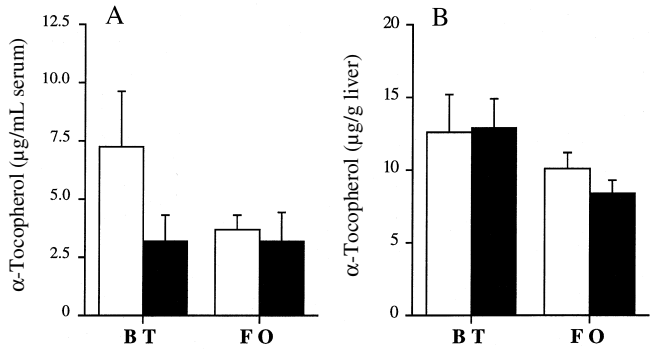

FIG. 3. Serum (A) and liver (B) α-tocopherol contents in mice fed BT or FO diets for 4 wk. Samples were taken 24 h after vehicle (open bar) or CCl_4 (solid bar) treatments. In the two-way ANOVA, there were effects of diet ($P < 0.005$), CCl_4 treatment ($P < 0.0005$), and the interaction between diet and CCl_4 treatment ($P < 0.005$) on serum α-tocopherol (A); and there was an effect of diet ($P < 0.0001$) on liver α-tocopherol (B). Each column with a bar represents the mean ± SD for seven to eight mice. See Figure 1 for abbreviations.

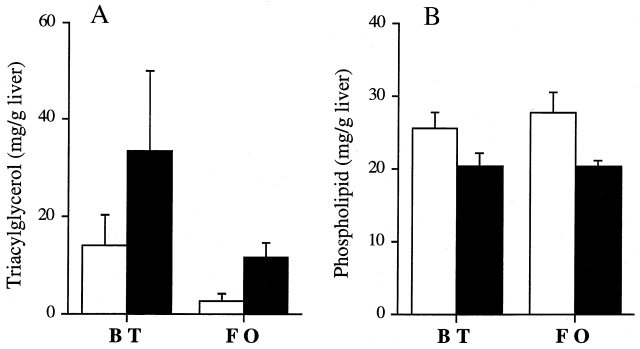

FIG. 5. Liver triacylglycerol (TG) (A) and phospholipid (PL) (B) contents of mice fed BT or FO diets for 4 wk. Samples were taken 24 h after vehicle (open bar) or CCl_4 (solid bar) treatments. Lipids were extracted from liver with chloroform/methanol, and TG and PL were separated by thin-layer chromatography on a silica gel and quantified by gas–liquid chromatography. In the two-way ANOVA, there were effects of diet ($P < 0.001$) and CCl_4 treatment ($P < 0.005$) but not of the interaction between diets and CCl_4 treatment on liver TG (A); and there was an effect of CCl_4 treatment ($P < 0.0001$) on liver PL (B). Each column with a bar represents the mean ± SD for five mice.

mg/mL in the BT diet group and 506 ± 269 mg/mL in the FO diet group, $n = 10$). There was no significant influence of CCl_4 treatment on the levels of either serum lipid (data not shown). In the vehicle-treated control groups, the liver TG concentration in the FO diet group was significantly lower than in the BT diet group (Fig. 5A). CCl_4 treatment significantly increased liver TG concentrations in both dietary groups. The interaction between the diet and CCl_4 treatment was not significant, although the level obtained by CCl_4 treatment in the FO diet group was still lower than in the BT diet group. In contrast, liver PL concentrations were significantly decreased by CCl_4 treatment in both dietary groups with no difference being observed between the two groups (Fig. 5B).

The fatty acid compositions of liver PL differed markedly between the FO and BT diet groups; the proportions of EPA (20:5n-3) ($P < 0.0001$) and DHA (22:6n-3) ($P < 0.0001$) were significantly increased whereas those of linoleic acid (18:2n-6) ($P < 0.0001$) and AA (20:4n-6) ($P < 0.0001$) were decreased in the FO diet group as compared with the BT diet group (Table 2). These differences in the proportions of PUFA led to a significant elevation of PI values in the FO diet group as compared with the BT diet group. Furthermore, notable changes associated with CCl_4 treatment were significant increases in the proportion of DHA ($P < 0.05$) and decreases in those of dihomo-γ-linolenic acid (20:3n-6) ($P < 0.0001$), AA ($P < 0.0001$) and EPA ($P < 0.005$), all eicosanoid precursors, in the liver PL of both dietary groups.

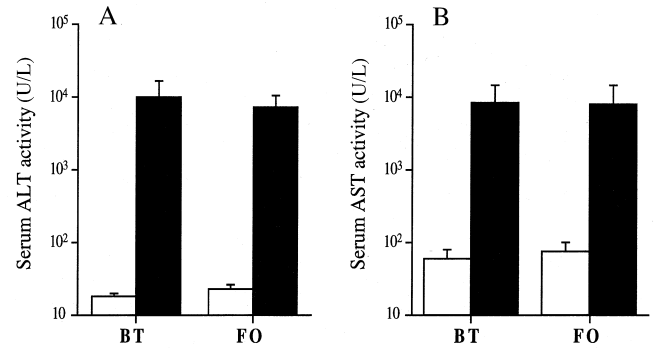

FIG. 4. Serum alanine aminotransferase (ALT) (A) and aspartate aminotransferase (AST) (B) activities in mice fed FO or BT diets for 4 wk. Samples were taken 24 h after vehicle (open bar) or CCl_4 (solid bar) treatments. In the two-way ANOVA, there was an effect of CCl_4 treatment ($P < 0.0001$) but not of diet on serum ALT (A) and AST (B) activities. Each column with a bar represents the mean ± SD for six to eight mice. See Figure 1 for abbreviations.

DISCUSSION

The TBARS assay detects mainly aldehydes formed as decomposition products of lipid peroxides (20). The conjugated diene assay relies on the rearrangement of 1,4-nonconjugated dienes to the conjugated diene form (19); this assay will also detect hydroxylipids having conjugated dienes (i.e., reduction products of lipid hydroperoxides). A more direct method for detecting PL hydroperoxides, a chemiluminescence-high-performance liquid chromatography method was developed, and by using this assay, significantly increased levels of phosphatidylcholine hydroperoxides were noted in livers of rats fed an FO diet (15) or administered CCl_4 (27). However, the values reported for serum PL hydroperoxides differ significantly among different laboratories (15,28), and the extent to which hydroperoxides produced during assays contribute to the measured values has not been evaluated accurately.

In the present study, dietary FO and CCl_4 treatment markedly increased liver TBARS contents measured in the absence of BHT in a synergistic manner, but no such marked effect was apparent in liver TBARS values measured in the presence of BHT and in conjugated diene contents (Figs. 1 and 2). These results suggest that significant amounts of BHT-

TABLE 2
Fatty Acid Compositions of Liver Phospholipids from Mice Fed the Beef Tallow (BT) or Fish Oil (FO) Diets (% of total fatty acids)[a]

	BT diet		FO diet			ANOVA		
Fatty acid	Control	CCl_4 treatment	Control	CCl_4 treatment	Pooled SD	Diet	CCl_4	Diet × CCl_4
16:0	17.1	20.4	23.1	23.9	3.1	<0.005	NS	NS
16:1	0.5	0.6	0.5	0.5	0.2	NS	NS	NS
18:0	20.0	20.7	18.9	20.4	1.8	NS	NS	NS
18:1	11.2	10.4	6.1	5.4	1.2	<0.0001	NS	NS
18:2n-6	16.6	15.7	11.0	11.1	1.5	<0.0001	NS	NS
18:3n-3	0.2	0.3	0.4	0.3	0.1	NS	NS	NS
20:3n-6	2.6	1.5	0.5	0.4	0.2	<0.0001	<0.0001	<0.0005
20:4n-6	14.5	12.0	11.1	9.1	1.0	<0.0001	<0.0001	NS
20:5n-3	1.1	0.9	4.6	2.3	0.2	<0.0001	<0.005	<0.01
22:5n-6	0.3	0.2	0.6	0.6	0.1	<0.0001	NS	NS
22:5n-3	n.d.	0.3	0.2	0.3	0.3	NS	NS	NS
22:6n-3	14.3	14.6	21.2	23.3	1.0	<0.0001	<0.05	NS
24:0	0.2	0.3	0.3	0.3	0.1	NS	NS	NS
24:1	0.9	0.9	0.9	0.9	0.2	NS	NS	NS
n-6	33.9	29.9	23.3	21.5	2.1	<0.0001	<0.01	NS
n-3	15.7	16.0	26.4	26.3	1.2	<0.0001	NS	NS
n-6/n-3	2.16	1.88	0.89	0.82	0.16	<0.0001	<0.05	NS
PI	203.6	193.8	259.6	256.0	8.6	<0.0001	NS	NS

[a]Values are means from five mice. n.d., not detected; NS, not significant ($P \geq 0.05$); PI, peroxidizability index; ANOVA, analysis of variance; CCl_4, carbon tetrachloride.

inhibitable artifacts (>90% of the values) are formed during the coloring reaction used for the original and most of modified TBARS assays. Furthermore, we suspect that lipid peroxides generated at higher rates *in vivo* in FO diet group might be cleared rapidly by endogenous antioxidants as well as antioxidative enzyme systems. Garrido *et al.* (5) have shown that liver microsomal membranes isolated from rats fed FO diet are more susceptible to *in vitro* oxidative stress induced by NADPH-ferric ion-ADP to produce greater amounts of TBARS. However, their study determined TBARS values in the absence of antioxidant in the assay, and isolated membranes are deficient of scavenger systems such as glutathione peroxidases, endogenous antioxidant vitamins, and their reducing enzyme systems present *in vivo*. Thus, the TBARS values measured in the absence of antioxidant as well as peroxidizability of isolated membrane preparations do not appear to reflect *in vivo* phenomena. A more specific, easy, and reliable assay for determining lipid peroxides present in tissues is still required, but our data indicate that liver lipid peroxides measured as TBARS with antioxidant BHT and as conjugated dienes are not increased by the CCl_4 treatment to levels which the difference in PI values of the diets predicts; more than a 10-fold difference in PI values of the diets resulted in less than a 1.15-fold difference in conjugated diene contents .

The fact that feeding highly autoxidizable PUFA decreases the concentrations of tissue α-tocopherol in animals (6) and humans (29) has been interpreted as being the consequence of oxidative consumption of α-tocopherol and has led to the suggestion for an increased dietary α-tocopherol requirement. However, serum α-tocopherol is transported by lipoproteins, and its concentration appears to be regulated both by the amounts and types of dietary fats (30,31). In addition, the tissue lipid contents also influence the tissue α-tocopherol levels (30,31). Therefore, the reduction of α-tocopherol concentrations in serum and liver by the FO diet (Fig. 3) might well be the consequence of decreased serum and liver lipid levels induced by FO (31). Furthermore, CCl_4 treatment caused no further reduction of α-tocopherol concentrations in either the serum or liver of mice fed the FO diet but did reduce serum α-tocopherol in the BT diet group (Fig. 3). We interpret that α-tocopherol status is not an indirect index for lipid peroxide tone in body but is more a reflection of lipid metabolism (i.e., the amounts of lipids in blood and tissues).

CCl_4 treatment induces TG accumulation in liver by inhibiting lipoprotein secretion (32). Although fatty liver was induced similarly in both the BT and FO diet groups, the liver TG concentration in the FO diet group was significantly lower than in the BT diet group, apparently indicating that the FO diet suppressed fatty liver induction by CCl_4 treatment. We have no evidence that easily autoxidizable DHA suppressed CCl_3·-induced liver injury, but most probably the relatively lower TG content in liver of the FO diet group is a consequence of decreased TG synthesis associated with preferential β-oxidation of DHA in peroxisomes (33).

Liver PL concentrations were reduced by CCl_4 treatment but not affected by dietary fatty acids (Fig. 5B). One might assume that the observed reduction of liver PL concentrations

by CCl_4 treatment is due to free radical-initiated degradation of PL enriched with autoxidizable PUFA. However, the decreases in liver PL concentrations were similar between the BT and FO diet groups (Fig. 5), despite significantly higher PI values of liver PL from the FO diet group (Table 2). In addition, it should be noted that no preferential decrease in DHA content was observed, but rather it increased, and dihomo-γ-linolenic acid, AA, and EPA all decreased in liver PL after CCl_4 treatment (Table 2). These observations indicate that mechanism(s) other than peroxidative consumption of PUFA account for the reduction of liver PL concentrations during CCl_4 treatment. Furthermore, the autoxidizabilities of PUFA estimated for air (4) appear not to be applicable to the observed changes in PUFA *in vivo* during CCl_4-induced liver injury. The amount of DHA, which is more easily autoxidizable than AA in air atmosphere, was relatively unchanged whereas that of AA was reduced by CCl_4 treatment. We suggest that CCl_4 treatment is toxic to PL-synthesizing enzymes and other enzymes in liver and may activate phospholipases to mobilize dihomo-γ-linolenic acid, AA and EPA, but that DHA is relatively stably enriched in PL under these conditions (Table 2). In fact, an *in vitro* study has indicated that AA is released upon exposure of hepatocytes to CCl_4, independent of free-radical generation by CCl_4 metabolism (34).

Needless to say, CCl_4 treatment under antioxidant deficiency leads to irreversible oxidative damage through lipid peroxidation as evidenced by elevated ethane exhaustion, an alternative measure of lipid peroxidation *in vivo*, and increased mortality in rats (35). In the presence of appropriate amounts of antioxidants and antioxidative enzyme systems, however, *in vivo* evidence is lacking that feeding highly autoxidizable PUFA such as α-linolenic acid, EPA, and DHA accelerates the development of oxidative stress-induced injury. Contrarily, many lines of evidence have indicated that long-term feedings of oils enriched in α-linolenic acid, EPA, or DHA suppress the so-called free radical diseases (i.e., aging, carcinogenesis, and atherosclerosis) as summarized elsewhere (36).

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Fatty Acid Carbon Isotope Ratios in Humans on Controlled Diets

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ABSTRACT: Carbon stable isotope ratios for six serum fatty acids (FA) are reported for human subjects on controlled fat diets to determine the range of natural isotope abundance and to demonstrate the leveling effect of a well-controlled diet. Twenty-nine subjects were randomly assigned to one of three controlled diets containing high, medium, or low fat. Diets were consumed for 8 wk. Serum samples were collected at baseline (0), 5, 6, 7, and 8 wk. FA were extracted and methylated. Isotope ratios were analyzed by high-precision gas chromatography combustion–isotope ratio mass spectrometry. At baseline, mean $\delta^{13}C$ for 16:0[b], 16:1[a], 18:0[a], 18:1[c], 18:2n-6[d], and 20:4n-6[bc] were –24.1, –21.7, –21.6, –25.6, –29.6, and –25.0‰, respectively, with an average standard deviation of 1.9‰. Most $\delta^{13}C$ decreased during the diet period and appeared to have stabilized by week 5 at –25.3, –21.9, –22.3, –26.5, –30.1, and –24.5‰, respectively. Between-subject variability decreased from 1.74 to 1.20‰ on the controlled diets. Measurement variability was 0.53‰. The within-subject variability during weeks 5–8 was 0.57‰ (range of 0.32–0.84‰), showing a minimum biological fluctuation on controlled diets. There was no diet group effect on $\delta^{13}C$ of serum FA. Except for 18:2, the $\delta^{13}C$ of experimental diets was lower than that of serum FA, consistent with observations in animals. These data show that carbon isotope ratios stabilize in response to controlled diets within 5 wk, reflecting the isotope ratio of their dietary source, and establish isotope ratio fluctuations for endogenous compounds for future studies.

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Stable isotopes are increasingly used for metabolic studies where radiotracers are undesirable and for studies involving natural variability where radiotracers are not applicable. Rapidly advancing high-precision mass spectrometry instrumentation and methods have particularly expanded the range of stable isotope nutritional studies. Familiar nutritional applications using high-precision tracer analysis include doubly labeled water for energy expenditure (1), labeled amino acids for protein turnover (2), and a variety of ^{13}C substrate-based breath tests (3–6). Elucidation of the human nutritional implications of stable isotope variation due to natural processes has been slow, but such information is widely used for ecological studies including diet studies in free-living animals, insects, and plants (7).

Thus far, few baseline $^{13}C/^{12}C$ studies have been reported; the few that have appeared are limited in subject number: serum cholesterol in 4 subjects (8) and in 1 subject (9), and urinary steroids (10) in 12 subjects. Mendelsohn *et al.* (8) evaluated the feasibility of using stable ^{13}C as a tracer for studying cholesterol metabolism *in vivo* by measuring ^{13}C content of commonly consumed foods and the stability of the ^{13}C content of serum cholesterol in four adults living in South Africa. They reported a very small difference between the carbon isotope composition of the diet and the cholesterol and no significant change of human serum cholesterol over time. Guo *et al.* (9) evaluated baseline fluctuations for stable isotope studies of human cholesterol metabolism. Becchi *et al.* (10) measured $^{13}C/^{12}C$ isotope ratio of urinary steroids to detect misuse of testosterone in sports. Their data suggest that the constant difference in $\delta^{13}C$ between precursor cholesterol and product testosterone can be exploited to detect the presence of exogenous synthetic testosterone, which generally has a $\delta^{13}C$ different from that of endogenous human testosterone, and thereby serves as a test for illicit use of performance-enhancing endogenous drugs.

The natural $^{13}C/^{12}C$ ratio in biological materials including food and tissues varies over about 1.09 ± 0.1 atom %. It is related to several factors, most prominently the mix of C_3 and C_4 plants from which dietary carbon is ultimately derived, the amount of marine food in the diet, the mixture of metabolic fuels, and the subject's physiological state (11). Background levels and their fluctuations must be established to permit calculation of excess isotope representing tracer. A recent study evaluating hydrogen and oxygen variability among waters showed that estimates of CO_2 production by the doubly labeled water method derive two-thirds of their variability from natural baseline fluctuations (3.23% of 4.90% coefficient of variation) (12). Real biological (between-subject) variability in any measured analyte contributes to overall error and must be taken into account in power calculations to establish subject numbers and dose sizes for tracer studies.

The recent wide availability of high-precision gas chromatography–combustion isotope ratio mass spectrometry

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Abbreviations: AA, arachidonic acid; AAD, Average American Diet; ALA, α-linoleic acid; DHA, docosahexaenoic acid; FA, fatty acid; FAME, fatty acid methyl ester; GCC–IRMS, gas chromatography combustion–isotope ratio mass spectrometry; LS, Very Low Saturated Fatty Acid Diet; PDB, Pee Dee Belemmite; SAS, statistical analysis system; S1, Step One.

(GCC–IRMS) facilitates stable isotope studies of individual biomolecules. Examples of human nutrition applications using artificial and natural tracers include work with fatty acids (FA) (3,14–16), and glucose kinetics (17).

Since normal FA $^{13}C/^{12}C$ fluctuations in free-living humans depend on dietary intake, minimum fluctuations can only be evaluated in subjects on a controlled diet. The relationship of dietary FA $^{13}C/^{12}C$ to plasma FA $^{13}C/^{12}C$ has yet to be established. Here, we report measurement of the $^{13}C/^{12}C$ ratio of six serum FA in 29 humans upon entry and during an 8-wk controlled diet trial to establish (i) the range of FA $^{13}C/^{12}C$ upon entry, (ii) the stabilization of FA $^{13}C/^{12}C$ after 5–8 wk on a controlled diet, (iii) the relationship of serum FA $^{13}C/^{12}C$ to dietary FA 13C/12C, and (iv) trends in the absolute serum FA $^{13}C/^{12}C$.

MATERIALS AND METHODS

Subjects and diets. Twenty-nine healthy adults, recruited as a part of the multicenter Dietary Effects on Lipoproteins and Thrombogenic Activity study described in detail elsewhere (18–20), were included in the current analysis. Briefly, subjects were randomly assigned to one of three controlled diets differing only in saturated fat content. The controlled diets were identical in all nutrients except for fat and were consumed for 8 wk. Diets were designed to provide 37, 30, and 26% of calories as fat with 16, 9, and 5% calories as saturated fatty acid, designated as Average American Diet (AAD), Step One (S1), or Very Low Saturated Fatty Acid (SFA) Diet (LS), respectively. Calories were made up with carbohydrate for diets S1 and LS, and all other components including monounsaturated FA at 14% of total calories, polyunsaturated FA at 7% of calories, and cholesterol, at 300 mg/d, were held constant. Diets were prepared by adding different amounts of various fats to otherwise low-fat meals. All fat sources were centrally procured in single lots that were used for the entire study to ensure uniformity. Fasting blood samples were collected before the study (0) and during weeks 5, 6, 7, and 8 of the controlled diet period.

Extraction and methylation. Serum was separated from red blood cells by centrifugation at 4°C and stored at −80°C. A 200-μL serum sample was saponified using 2 mL of 0.5 N KOH in ethanol with heating at 60°C for 1 h (21). After cooling to room temperature, nonsaponifiables were removed by washing twice with 2 mL of hexane, and internal isotopic standard heptadecanoic acid was added. The samples were then acidified with 1 mL of 1 N HCl, and 2 mL hexane was added. The total lipids were extracted by taking the upper hexane layer (22). Fatty acid methyl esters (FAME) were prepared by adding 1 mL 14% BF_3 in methanol and heating at 100°C for 4 min. Hexane (2 mL) was then added and the tubes vortexed. After 2 mL of saturated NaCl solution was added, tubes were centrifuged at 3,000 rpm, and the top hexane layer containing the FAME was transferred to another tube. The FAME were dried under N_2 and resuspended in a measured amount of hexane for analysis. All serum samples were split into two aliquots, which were extracted, treated, and analyzed in parallel. Replicate aliquots were used to assess measurement error reflecting total error for extraction, methylation, and instrument error.

Homogeneous aliquots of each of the three diets were prepared for FA isotope ratio analysis (23,24). Eight daily menus, representing a complete diet cycle, were frozen and shipped to the Food Analysis Laboratory Control Center at Virginia Tech University. Each eight-menu sample was composited and individual aliquots removed for fatty acid isotopic analysis. Samples of 0.1–0.2 g were extracted with 10 mL total of solvent in duplicate, and were prepared using the same method as for serum.

Isotopic analysis and calculations. FAME prepared from 16:0, 16:1, 18:0, 18:1, 18:2n-6, and 20:4n-6 were analyzed by GCC–IRMS, which has been described in detail elsewhere (25). Briefly, a Varian 3400 GC (Varian Analytical Instrument, San Fernando, CA) was connected to an 850°C combustion furnace linked to a Finnigan MAT 252 (Bremen, Germany) high sensitivity, high-precision IRMS for $^{13}C/^{12}C$ ratio analysis. The GC was equipped with a J&W DB-23 (60 m × 0.32 mm i.d., 0.25 μm film thickness) column. The combustion furnace was nominally held at 850°C and contained oxidized Cu wire and Pt as a catalyst. The GC effluent was directed by He carrier gas into a combustion furnace where organic compounds were quantitatively converted into CO_2 and H_2O. The water was trapped in a Nafion (DuPont, Wilmington, DE) tube, and only CO_2 gas was admitted to the ion source of a Finnigan 252 IRMS, with dedicated detectors for three major ions, m/z = 44, 45, and 46, and absolute sensitivity of about 103 molecules/ion detected. The isotope ratio $^{13}C/^{12}C$ was calculated using the m/z = 46 to correct the contribution of ^{17}O-substituted CO_2 appearing at m/z = 45. The analytical precision of replicate measurements is <0.3‰.

The standard notation for expression of high-precision gas IRMS is the $\delta^{13}C$ notation defined as in Equation 1.

$$\delta^{13}C = \left(\frac{R_{SPL} - R_{PDB}}{R_{PDB}}\right) \times 1000 = \left(\frac{R_{SPL}}{R_{PDB}} - 1\right) \times 10 \qquad [1]$$

where $R_x = {}^{13}C/{}^{12}C$ and SPL refers to the sample and PDB refers to the international standard Pee Dee Belemnite, R_{PDB} = 0.0112372. Most PDB was chosen as a standard partly because it is relatively rich in ^{13}C; the carbon of most biological compounds is depleted in ^{13}C relative to this standard owing to fractionation during photosynthesis so that most compounds are described with a negative $\delta^{13}C$. The notation emphasizes that high precision is achieved as a relative measure, and has the convenient feature of eliminating several leading, unchanging digits. For example, R = 0.0112372 and R = 0.0112260 correspond to $\delta^{13}C$ = 0‰ and −1‰, respectively. Interpretation is considerably simplified as the analytical precision is typically SD ($\delta^{13}C$) < 0.4‰, and most biological compounds derived from contemporary life are in the $\delta^{13}C$ = −5 to −40 range.

TABLE 1
$\delta^{13}C$ of Six Serum Fatty Acids from 29 Free-Living Adults[a]

Fatty acid	$\delta^{13}C$ Mean	SD
16:0	–24/07[b]	1.74
16:1	–21.68[a]	2.37
18:0	–21.64[a]	1.10
18:1n-9	–25.59[c]	1.59
18:2n-6	–29.63[d]	1.58
20:4n-6	–24.98[b,c]	2.77
Average	–24.60‰	1.86‰

[a]Different superscripts (a–d) indicate significant difference at the level of $\alpha = 0.05$.

Carbon within a molecule is converted to CO_2 prior to analysis so that all positions contribute to measured isotope ratio. Derivative carbon isotope ratios reflect both the isotopic composition of the reagent and any kinetic isotope effects associated with the derivatization reaction. This latter effect mandates the use of an empirical internal standard since the reagent carbon isotope ratio cannot be taken as representative of the derivative carbon. This effect is implicitly taken into account in tracer studies where a baseline isotope ratio is subtracted. However, for studies of absolute isotope ratio such as this, an internal isotopic standard is required. FA $\delta^{13}C$ (‰) were corrected using C17:0 with known isotope ratio as reported previously (26). When no artificially enriched tracers are involved, a mass balance equation can be used for the isotope ratio of the derivative methyl group as described previously (15), in Equation 2.

$$n_{FAME}\,\delta^{13}C_{FAME} = \delta^{13}{}_{Me} + n_{FA}\,\delta^{13}C_{FA} \qquad [2]$$

where n_{FA} is the moles of C per mole FA, and the subscripts Me, FA, and FAME refer to the methyl group, the FA, and the measured FAME, respectively. $\delta^{13}C$ for the methyl group is assumed to be uniform for all FAME and identical to that for the internal standard Me17:0.

Statistical analysis. The Statistical Analysis System, SAS 6.11 (SAS Institute, Cary, NC) was used for regression and analysis of variances. The mixed model procedure was employed for assessment of within-subject, biological (between-subject), and measurement variability. Tukey's pairwise comparison at the level of $\alpha = 0.05$ was used for comparing means among treatments in Minitab Release 11.11 (Minitab, Inc., State College, PA).

RESULTS

FA $^{13}C/^{12}C$ upon entry. Means and standard deviations in $\delta^{13}C$ (‰) units for six serum FA in 29 subjects upon entry into the study are presented in Table 1. At baseline, the mean $\delta^{13}C$ ranged from –29.6 for 18:2 to –21.64 for 18:0, respectively, with an average of –24.60‰. The average standard deviation was 1.86‰ for all six FA. Since the overall analytical error for workup and isotopic analysis is <0.6‰, the predominant factor in the standard deviation is real biological variability.

Time and diet effect. The mean $^{13}C/^{12}C$ ratios of six serum FA were similar for all three diet groups; for brevity Table 2 summaries the data for S1 Diet only. There are significant and persistent differences between FA. In this group, only the last 18:1 isotope ratio is significantly different from the baseline, which may reflect the relatively large pool of this FA. Figure 1 presents a plot of the ^{13}C content of serum FA in group AAD from 0 through 8 wk to illustrate the effects of continuing diet consumption. The ^{13}C content of serum FA changed substantially from week 0 to week 5 and then stabilized through week 8; plots from diet groups S1 and LS were qualitatively similar. From the analysis of variance, there was a significant time effect in each of the six FA, indicating the controlled diets did alter the $^{13}C/^{12}C$ of serum FA.

To test the hypothesis that the isotope ratios were stable in the latter part of the diet period from weeks 5 through 8, a linear regression was performed using each of the six FA isotope values for weeks 5–8 for each subject with the resulting significance of the slopes evaluated. Only 14 of the 174 slopes, or about 8%, were significantly different from 0 at the

TABLE 2
Mean $\delta^{13}C$ of Six Serum FA over Time (0–8 wk) in Group S1[a]

Time (wk)	Serum FA in Diet S1 16:0	16:1	18:0	18:1	18:2	20:4
0	–24.57[a,2]	–21.61[a,1]	–22.03[a,1]	–25.38[a,2]	–29.73[a,3]	–24.69[a,2]
	(1.21)	(2.68)	(0.92)	(2.06)	(1.86)	(2.95)
5	–25.01[a,3]	–21.10[a,1]	–22.42[a,2]	–26.35[a,b,4]	–30.19[a,5]	–23.89[a,3]
	(0.85)	(1.71)	(1.01)	(0.68)	(1.55)	(1.98)
6	–25.11[a,2]	–21.81[a,1]	–22.52[a,1]	–26.40[a,b,3]	–30.04[a,4]	–24.09[a,2]
	(0.95)	(1.79)	(0.96)	(1.35)	(1.67)	(2.05)
7	–24.81[a,2]	–21.25[a,1]	–22.41[a,1]	–26.04[a,b,3]	–30.07[a,4]	–23.32[a,2]
	(0.72)	(1.56)	(0.94)	(1.13)	(1.56)	(1.96)
8	–25.11[a,2]	–22.22[a,1]	–22.60[a,1]	–26.67[b,3]	–30.08[a,4]	–24.32[a,2]
	(0.83)	(1.62)	(1.48)	(1.02)	(1.60)	(2.20)

[a]Groups Average American Diet (AAD) and Very Low Saturated Fatty Acid Diet (LS) were similar. Each number in parentheses reflects standard deviation of 10 subjects in the diet group. Different letter superscripts (a,b) indicate significant difference among times at the level of $\alpha = 0.05$; different number superscripts (1–5) indicate significant difference among fatty acid (FA) at the level of $\alpha = 0.05$. S1, Step One Diet.

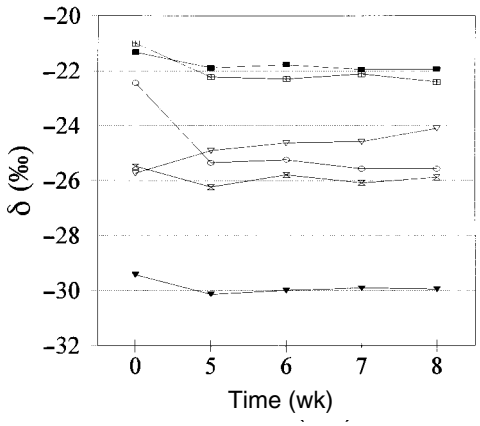

FIG. 1. $^{13}C/^{12}C$ in $\delta^{13}C$ (‰) units of serum fatty acid in diet group average American diet measured upon study entry, assigned week 0, through 8 wk. Overall error averages 0.56‰. Symbols: ○, 16:0; □, 16:1; ■, 18:0; ⧖, 18:1; ▼, 18:2; ▽, 20:4.

$P < 0.05$ level, indicating that most FA had stabilized by weeks 5–8. Original data on slopes and their significance can be found elsewhere (27). Serum FA data for weeks 5 through 8 were pooled for further statistical analyses.

Analysis of variance was performed to investigate the diet treatment effect in stabilizing the isotope ratio. There was no significant diet group effect in stabilization of ^{13}C content in serum FA except 18:1. For 18:1, group AAD is different from group C; however, the difference as shown in Table 2 was very small, less than 1‰. Therefore, serum FA data for all three diet groups were combined for further statistical analyses unless otherwise specified.

Measurement, between-subject, and biological variability. Intersubject, intrasubject, and measurement variability were evaluated using the mixed model procedure in SAS, and results are summarized in Table 3. Average between-subject variability was 1.74‰ at week 0, and decreased to 1.20‰ for weeks 5–8. Subject variability decreased by an average of about 0.5‰ in the controlled diet period in all FA except 18:2, showing that the uniform isotope ratio of the diet FA induced the isotope ratios to converge in most cases. The convergence of isotope ratios on controlled diets is illustrated in Figure 2, which shows results for 16:0. The top histogram shows the distribution of isotope ratios upon entry to the study, which range from −28 to −21‰. Figure 2B shows the aggregate isotope ratios for weeks 5–8, which range from −27 to −23‰.

Measurement variability represents variability between replicate measurements which included extraction, FAME preparation, and imprecision of GCC–IRMS instrument measurement. Table 3 presents measurement variability for six serum FA evaluated at entry and for weeks 5–8, and averaging about 0.56‰. Overall, measurement variability was smallest for 16:0, which is present in high concentration and gives a sharp peak because of its early elution time. Consistent with this, measurement variability was greatest for 20:4, which is a minor FA eluting last of those reported.

Within-subject (biological) variability for weeks 5–8 averaged 0.57‰ which can be taken as a minimum baseline fluctuation in the ^{13}C content of serum FA on controlled diets.

Serum and dietary FA. The FA ^{13}C content in representative, homogenized aliquots of the three experimental diets was analyzed. The results are summarized in Table 4. No significant differences were detected among the diets for 18:0, 18:1, or 18:2. Statistically significant differences were observed in 16:0 of <2‰ and 16:1 of <0.4‰, which may be due to sampling error, and no data were obtained for 20:4 since its concentration was extremely low.

The mean ^{13}C content of serum FA before and during the dietary period is plotted along with the diet levels in Figure 3. The ^{13}C content of the diet FA was lower than that of serum FA for 16:0, 16:1, 18:0, and 18:1, but not 18:2. Isotope ratios of serum FA decreased during the controlled dietary period, indicating that isotope ratios of serum FA were approaching those of dietary FA. The ^{13}C content of 18:2 in diet was about 2‰ higher than that of serum suggesting that the ^{13}C content of this essential FA was different from that of nonessential FA.

DISCUSSION

The major findings in this study are (i) serum FA carbon isotope ratios stabilize within 5 wk in adults on controlled diets, (ii) measurement variability including extraction and derivatization was about 0.56‰, (iii) variability attributable to subjects decreased from a mean of 1.74‰ upon entry to 1.20‰ on the controlled diets, indicating a significant narrowing of isotope ratio under controlled dietary conditions, and (iv) mean FA carbon

TABLE 3
Summary of Intersubject, Measurement, and Intrasubject Variability ‰

	Between subject		Measurement		Within subject
Fatty acid	wk 0	wk 5–8	wk 0	wk 5–8	wk 5–8
16:0	1.38	0.71	0.45	0.35	0.32
16:1	2.28	1.35	0.75	0.69	0.84
18:0	1.09	0.94	0.42	0.51	0.53
18:1	1.51	0.80	0.58	0.45	0.53
18:2	1.50	1.60	0.64	0.50	0.50
20:4	2.70	1.78	0.88	0.54	0.71
Average	1.74‰	1.20‰	0.62‰	0.51‰	0.57‰

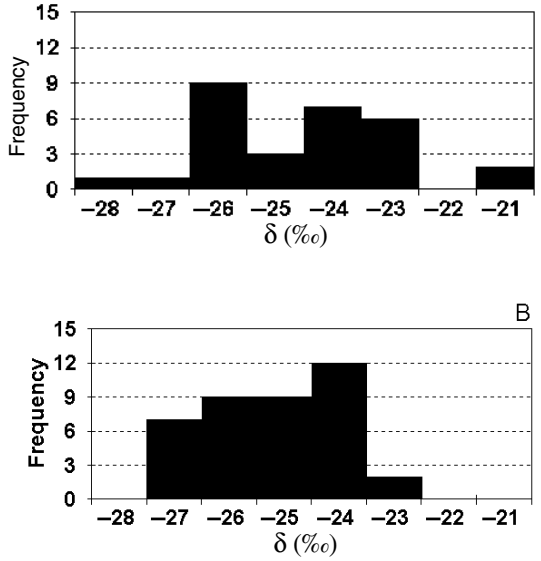

FIG. 2. (A) Histogram showing the distribution of $^{13}C/^{12}C$ of serum 16:0 from the 29 subjects upon entry to the study. (B) Histogram showing $^{13}C/^{12}C$ of serum 16:0 averaged over weeks 5–8. The distribution has narrowed considerably on the controlled diets.

isotope ratios are related to that of their dietary source and are enriched in ^{13}C for nonessential FA, as expected; surprisingly, they are depleted for the only essential FA measured in this work, linoleic acid.

There was no change in the ^{13}C content of serum 18:2 on the controlled diets. Since 18:2 is an essential FA, the overwhelming percentage of serum 18:2 is derived directly from the diet, with very small amounts synthesized from 16:2 (28). It can therefore be concluded that the controlled diet 18:2 isotope ratio was nearly identical to that of the average uncontrolled diet. All dietary 18:2 is ultimately derived from vegetable oils, since 18:2 in animal foods originates in plants. This observation therefore suggests a surprising dietary homogeneity of vegetable sources. An alternative hypothesis supported by observation of gross isotope ratios in pigs is that there is a greater response to a more negative $\delta^{13}C$ diet than to a more positive $\delta^{13}C$ diet compared to $\delta^{13}C$ of body FA. In pigs, the change of tissue $\delta^{13}C$ when diets were switched over from C_3 to C_4 plants (low to high $\delta^{13}C$) was much smaller than that from C_4 to C_3 (high to low $\delta^{13}C$), although there are no known mechanisms for this difference (29).

The qualitative relationship of stable isotope ratio between diet and serum FA was similar in all nonessential FA, where the isotopic distribution is a function of the relative contributions of *de novo* synthesis and assimilation from the diet. Limited information is available on turnover rates in tissues and specific biochemicals in large animals. It has been reported that fat equilibrates with diet more slowly than other major compounds such as lactose, casein, and whey of cow's milk when the dietary isotope ratio is switched (30,31). No data on specific FA have been reported.

In vivo studies of human and animal metabolism using ^{13}C-labeled substrates are commonly used to derive several types of information such as absorption and oxidization of dietary nutrients, biosynthesis and turnover of biochemicals, assessment of disease status, and observed activity of specific enzymes (3,32). The present data represent the most extensive study of baseline $^{13}C/^{12}C$ in specific biocompounds under controlled conditions, and can be used in power calculations to inform design of kinetic modeling studies using ^{13}C-labeled substrates.

Fry and Sherr (33) summarized studies of the relationship between animal tissue isotope ratio and their foods. Most studies that compared whole body or tissue isotope ratios found that $\delta^{13}C$ values are within ± 2‰ of the diets (33). A study of small animals such as *Musca* and *Caliphora* fed horsemeat or pork showed enrichments of 1.5–3.0‰ in tissue lipids compared to dietary lipids (34). Similar results were obtained for carbohydrates, but only a 1‰ enrichment was observed for protein fractions. Although there is no clear biochemical mechanism to explain these results, the well-known depletion of breath CO_2 of about 2‰ compared to dietary sources suggests that ^{12}C is preferentially catabolized leaving behind ^{13}C-enriched compounds to assimilate into tissue (34–37). This general mechanism would explain our results, since nonessential FA are composed of the preformed dietary FA that escape oxidation as well as FA synthesized *de novo* from acetate from partially oxidized glucose. This does not explain why serum 18:2 is 3‰ depleted in ^{13}C compared to dietary 18:2, for which we can only speculate that ^{12}C is discriminated against at the first committed step in β-oxidation.

TABLE 4
Average $\delta^{13}C$ of Fatty Acid in the Three Diets, and Serum Fatty Acid 0 and wk 5–8[a]

Diet group	Fatty acid					
	16:0	16:1	18:0	18:1	18:2	20:4
AAD	−25.93[a]	−23.92[a]	−23.40[a]	−26.64[a]	−27.49[a]	n.d.[a]
S1	−25.38[a]	−25.26[b]	−23.96[a,b]	−26.92[a]	−27.05[a]	n.d.
LS	−26.51[b]	−25.85[b]	−24.54[b]	−27.57[b]	−27.09[a]	n.d.
Diet average	−25.94	−25.01	−23.96	−27.04	−27.21	n.d.
Serum–wk 0[b]	−24.07[2] (1.74)	−21.68[1] (2.37)	−21.64[1] (1.10)	−25.59[3] (1.58)	−29.63[4] (1.58)	−24.98[2,3] (2.77)
Serum–wk 5–8[b]	−25.25[3] (0.85)	−21.86[1] (1.72)	−22.33[1] (1.17)	−26.51[4] (1.12)	−30.14[5] (1.65)	−24.53[2] (2.00)

[a]n.d., not determined. See Table 2 footnote for explanation of superscript numbers and letters and for other abbreviations.
[b]Values in parentheses represent standard deviation.

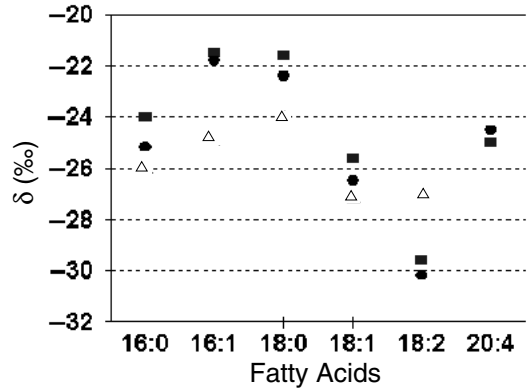

FIG. 3. Comparison of $^{13}C/^{12}C$ between serum fatty acids at week 0 (■), average of weeks 5–8 (●), and diet (△).

Studies of whole body and tissue isotope ratio are routine methods for establishing food-web relationships in terrestrial and aquatic ecology (e.g., Ref. 38), which focus on identifying food sources using isotope relationships. For instance, the foods of animals living in seagrass meadows may be distinguished since offshore foods ultimately derive their carbon from phytoplankton, with $\delta^{13}C = -21‰$, while native seagrasses and algae have $\delta^{13}C = -10$ to $-14‰$. Schoeller *et al.* (39) have discussed the application of a similar strategy to determination of the North American human food web, and have found carbon isotope abundances in several tissues to be accurate predictors of carbon derived from C_4 plants.

In ongoing studies, Haggarty *et al.* are applying this strategy to the study of human food sources (40). Docosahexaenoic acid (DHA) can be consumed preformed in marine foods, or can be synthesized from its precursor FA α-linolenic acid (ALA) normally derived from terrestrial sources. Marine food sources are generally depleted in ^{13}C relative to terrestrial food sources. Thus, a comparison of the ^{13}C content of DHA and ALA should be similar in humans consuming diets principally of terrestrial origin, whereas humans consuming larger proportions of their diets as marine foods would cause the $\delta^{13}C$ of DHA and ALA to diverge. Measurements of breast milk FA from lactating omnivore mothers in Scotland showed that most DHA was synthesized, indicating a low marine consumption in that particular population.

Natural variability in carbon isotope abundance can be purposefully exploited for low-enrichment tracer studies. A recent study of arachidonic acid (AA) synthesis from linoleic acid precursor was performed by switching breast-fed or formula-fed infants to a corn oil-based formula (14) and following the increase in AA isotope ratio over time; the data facilitated an estimate that 23% of plasma AA was synthesized from corn oil-derived linoleic acid consumed in the previous 4 d.

In conclusion, this study shows that $^{13}C/^{12}C$ isotope ratios stabilize in response to controlled diets by week 5. Serum FA take on isotope ratios reflecting their dietary source, with some modification related to metabolic activity for each FA. Metabolic differences between essential and nonessential FA may affect the ^{13}C content of serum FA. Biological fluctuation of 0.57‰ during controlled diet period (weeks 5–8) suggests a minimum $\delta^{13}C$ fluctuation of serum FA for human FA metabolism study.

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Dietary Fatty Acid Profile Affects Endurance in Rats

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ABSTRACT: Typically, athletes are advised to increase their consumption of carbohydrates for energy and, along with the general population, to reduce consumption of saturated fats. It is now recognized that fats are not identical in their influence on metabolism, and we argue that the composition of the polyunsaturated fat component should not be ignored. The aim of this study was to manipulate the dietary fatty acid profile in a high-carbohydrate diet in order to investigate the effect of dietary polyunsaturates on submaximal endurance performance in rats. Rats were fed one of three isoenergetic diets containing 22 energy percentage (E%) fat for 9 wk. The diets comprised an essential fatty acid-deficient diet (containing mainly saturated fatty acids); a diet high in n-6 fatty acids, High n-6; and a diet enriched with n-3 fatty acids, High n-3. Submaximal endurance in rats fed the High n-3 diet was 44% less than in rats fed the High n-6 diet ($P < 0.02$). All rats were then fed a standard commercial laboratory diet for a 6-wk recovery period, and their performances were reevaluated. Although endurance in all groups was lower than at 9 wk, it was again significantly 50% lower in the High n-3 group than the High n-6 group ($P < 0.005$). Although n-3 fats are considered beneficial for cardiovascular health, they appear to reduce endurance times, and their side effects need to be further investigated.
Lipids 32, 1265–1270 (1997).

A vast literature (and indeed a vast industry) advises athletes to consume high-carbohydrate diets in order to maximize physical performance. In doing so, athletes have often been advised to reduce their consumption of fats. This seems to stem from studies of cardiovascular disease where advice is that patients should reduce their total fat consumption while increasing their consumption of n-3 fats, but these recommendations for athletes have little comprehensive research support. However, fats are at least as physiologically important and their metabolism is as complex as carbohydrates. Clearly, the consequences and side effects of this changed dietary profile in humans need to be investigated.

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Abbreviations: ANOVA, analysis of variance; EDL, extensor digitorum longus; EFAD, essential fatty acid-deficient; E%, energy percentage; High n-6, diet high in n-6 fatty acids; High n-3, diet high in n-3 fatty acids; PUFA, polyunsaturated fatty acid; S,M,P_{n-6},P_{n-3}, ratio of saturated to monounsaturated to n-6 PUFA to n-3 PUFA; $\dot{V}O_2$max, maximal rate of oxygen consumption.

Although classical studies have led to the concept of carbohydrate loading for optimal physical performance (1), at least six articles published since 1983 have shown that performance in rats (2–4), dogs (5), and humans (6,7) may actually be improved when a high-fat diet is consumed for a short time. Surprisingly, the effects on endurance of different types of fats (whether saturated or unsaturated) have not been investigated, even though they have clearly been shown to have a multitude of physiological effects. The few studies which have investigated the effects of dietary fat manipulation on physical performance (8–10) have shown that altered levels of n-3 fatty acids do not affect maximal oxygen consumption, but none of these studies has investigated possible changes in endurance. Although the current dogma is that the diets of endurance athletes should be high in carbohydrates, we argue that the fat component should not be ignored. Specifically, the classes of fatty acids should not be treated identically because (i) different fatty acids are oxidized at different rates (11) and (ii) we have recently shown that a diet rich in saturated fats is detrimental to endurance performance compared to diets rich in unsaturated fats or carbohydrates (12).

This study was a part of a larger study examining the effects of changes in dietary essential polyunsaturated fats on muscle phospholipid composition (13), isolated muscle performance *in vitro* (14), and physical performance of whole animals. For 9 wk, rats were fed one of three isoenergetic test diets which differed only in their fatty acid composition. The diets were (i) deficient in the essential fatty acids, EFAD; (ii) high in n-6 fatty acids, High n-6; and (iii) enriched with n-3 fatty acids, High n-3. Following this, the rats were fed a commercial stock diet in order to determine the reversibility of any dietary effects on endurance.

We now report the effects of these same dietary fatty acid manipulations on the endurance of rats, as well as the extent to which these dietary effects were reversed after returning animals to the stock diet.

MATERIALS AND METHODS

Animals and diets. All experiments were approved by the University of Wollongong's Animal Experimentation Ethics Committee. Male weanling Wistar rats, bred at the University of Wollongong (housed individually at 22 ± 2°C and 57 ± 2% relative humidity, age 21–23 d, mean weight of 54 ± 1 g) were

randomly assigned, one per litter, to each of three dietary groups. Groups of eight rats were maintained on each test diet for 9 wk. Endurance was then assessed using a graded submaximal treadmill running test. Following this test, all rats were fed a common rat commercial diet [Allied Rat and Mouse Cubes (mean energy composition 12 E% fat, 64 E% carbohydrate, and 24 E% protein), Fielders' Agricultural Products, Tamworth, Australia)] for a 6-wk "recovery" period. At all times, rats were given free access to food and water, and food intake and body mass were recorded throughout the study.

All test diets were identical except for their lipid composition (22 E% fat, 56 E% carbohydrate, 22 E% protein). They contained (g/kg) sucrose 560, protein (casein) 220, oil 100, water 50, mineral mix 50, vitamin mix 10, cellulose 10. Details of the mineral mix and vitamin mix as well as of the methods of lipid analysis used are given in Ayre and Hulbert (13). The EFAD diet contained 100 g/kg coconut oil (ETA Food Services, Wollongong, Australia) as the only source of fat and was therefore lacking both n-6 and n-3 essential polyunsaturated fatty acids (PUFA) (the ratio of saturated to monounsaturated to PUFA, S,M,P_{n-6},P_{n-3} ratio, was 95:4:1:0). The High n-6 diet contained 100 g/kg sesame oil (Meadowlea Foods, Sydney, Australia) which is high in n-6 fatty acids; in this diet the S,M,P_{n-6},P_{n-3} ratio was 16:30:50:4. The High n-3 diet contained 70 g/kg sesame oil and 30 g/kg Max EPA oil (R.P. Scherer, Melbourne, Australia) containing 30% n-3 fatty acids. It was therefore enriched with n-3 PUFA and in this diet the S,M,P_{n-6},P_{n-3} ratio was 21:25:35:16. The S,M,P_{n-6},P_{n-3} ratio of the rat commercial diet was 33:32:30:2. The specific fatty acid content of the test diets and the stock diet is given in Ayre and Hulbert (13). In another group of rats fed the same diets, the fatty acid composition of phospholipids extracted from both the soleus and extensor digitorum longus (EDL) muscles was determined. The methods are presented in Reference 13.

Testing program. We assigned a cryptic code number to each rat, and endurance tests were performed so that the dietary group of each animal was concealed from the tester. Rats were fasted overnight and all littermates were tested in random order on the same day toward the end of the light cycle. Submaximal endurance was assessed while rats ran on a single-lane motor-driven treadmill. In the week prior to each testing period (i.e., after both 8 wk on the test diets and 5 wk on the commercial diet), rats underwent a habituation program (15). Each rat was given a 5-min exercise period with treadmill speed and incline increasing daily to 25.2 m/min and 10°, respectively. Endurance tests were then performed both after 9 wk on the three test diets and following 6 wk recovery on the commercial stock diet, following the protocol of Baldwin *et al.* (15). Rats commenced running at 12 m/min speed, 0° incline for 3 min, after which the treadmill was raised 2° and speed was increased to 27 m/min and held constant. Thereafter, the angle of incline was increased by 2° every 10 min. We measured time to exhaustion during treadmill running in order to provide an estimate of endurance. The length of time each rat could keep running at this workload was recorded. To ensure that rats ran to exhaustion, we routinely stimulated them with jets of compressed air while they were running free on the treadmill. A rat was considered to have reached exhaustion when it positioned itself at the back of the chamber for 10 s or more and no longer responded to repeated jets of compressed air. The amount of work performed by each rat was calculated as the product of body mass (i.e., force in kiloponds), distance traveled, and percentage grade (i.e., the product of the sine of the treadmill angle and 100), as described by Powers and Howley (16).

Statistical methods. All values are expressed as mean ± SEM. The *a priori* alpha level was 0.05. A one-way analysis of variance (ANOVA) was used to test for heterogeneity of food intake and rat weights. To test for significant differences in endurance and work between the dietary groups, we used the statistical package, SAS (SAS Institute, Cary, NC: 1979) to perform Model lll two-factor ANOVA without replication; the fixed factor was diet and the random factor was litter and/or day of testing. Since only one rat per litter was allocated to each treatment and each litter was tested on a different day, it is not possible to determine whether significant effects of this factor are litter effects as such, or the product of day-to-day variation in experimental conditions. However, the design ensures that variation among litters and days of testing does not obscure the effects of the diets. Two separate ANOVA were performed: after 9 wk on the test diets and after 6 wk on the common diet. Wherever ANOVA revealed significant effects of diet, we used Tukey's studentized range test to determine which dietary groups produced significantly different responses (17).

RESULTS

There was no significant effect of diet on body mass, either after the 9-wk experimental period (EFAD = 363 ± 20 g; High n-6 = 387 ± 14 g; High n-3 = 405 ± 5 g), or after 6 wk on the common diet (EFAD = 411 ± 24 g; High n-6 = 445 ± 14 g; High n-3 = 463 ± 7 g). There were significant effects of the diets on the fatty acid composition of muscle membranes (see Table 1). The percentage of total unsaturates in membranes was not influenced by diet, but the relative content of the different types of unsaturates was dramatically influenced by diet. For example, the different diets resulted in a 10-fold range in the n-3 polyunsaturate content of muscle phospholipids. The influence of diet was similar for both the fast (EDL) and the slow (soleus) muscle types.

Almost all rats ran willingly and there was surprisingly little variation within dietary groups (Fig. 1). The overall endurance performance for all groups decreased between the two endurance tests by approximately 50%. Dietary manipulation had a highly significant effect on exercise performance (Fig. 1). The endurance of both the High n-3 and EFAD groups was markedly lower than that of the High n-6 group both after 9 wk on the test diets and after 6 wk on the common diet. After the 9-wk experimental period, rats on the

TABLE 1
Effects of Dietary Fatty Acid Profile on Phospholipid Fatty Acid Composition (g/100 g fatty acids) of Soleus and Extensor Digitorum Longus Muscles of Wistar Rats[a]

Fatty acid	Soleus[b]			Extensor digitorum longus[b]		
	EFAD	High n-6	High n-3	EFAD	High n-6	High n-3
16:0	10.2 ± 1.8	13.2 ± 0.6	14.2 ± 1.0	23.9 ± 0.7	21.8 ± 1.0	21.6 ± 2.2
16:1n-9	5.3 ± 0.5^{a}	0.9 ± 0.4^{b}	0.7 ± 0.4^{b}	6.4 ± 0.6^{a}	0.2 ± 0.2^{b}	0.3 ± 0.2^{b}
18:0	21.9 ± 1.8	19.4 ± 1.6	20.0 ± 1.3	13.6 ± 0.7^{a}	15.9 ± 0.3^{a,b}	17.6 ± 1.0^{b}
18:1n-9	15.6 ± 1.4^{a}	8.4 ± 1.7^{b}	6.0 ± 0.5^{b}	21.6 ± 0.5^{a}	5.9 ± 0.3^{b}	17.6 ± 1.0^{b}
18:1n-7	4.2 ± 0.7	3.1 ± 0.2	2.9 ± 0.3	3.3 ± 0.1^{a}	2.1 ± 0.1^{b}	2.2 ± 0.2^{b}
18:2n-6	9.2 ± 1.0^{c}	20.1 ± 1.4^{a}	15.4 ± 1.0^{b}	6.8 ± 0.5^{a}	19.1 ± 1.3^{b}	16.3 ± 0.6^{b}
20:3n-9	9.6 ± 0.3^{a}	0.1 ± 0.1^{b}	—^{b}	6.1 ± 0.7^{a}	0.1 ± 0.0^{b}	—^{b}
20:3n-6	0.9 ± 0.1	0.4 ± 0.2	0.5 ± 0.2	0.5 ± 0.2^{a}	0.2 ± 0.2^{a,b}	—^{b}
20:4n-6	17.7 ± 2.1^{b}	23.0 ± 1.2^{a}	10.4 ± 0.4^{c}	11.3 ± 0.7^{a}	19.9 ± 2.0^{b}	7.2 ± 0.4^{c}
20:5n-3	—^{b}	—^{b}	1.1 ± 0.4^{a}	0.3 ± 0.2	0.1 ± 0.1	0.3 ± 0.2
22:4n-6	1.1 ± 0.2^{a,b}	2.4 ± 0.6^{a}	—^{b}	0.9 ± 0.1^{a,b}	2.0 ± 0.6^{a}	—^{b}
22:5n-6	1.2 ± 0.7^{b}	3.8 ± 0.5^{a}	—^{b}	2.0 ± 0.1^{a,b}	2.8 ± 0.8^{a}	—^{b}
22:5n-3	0.2 ± 0.1^{b}	1.6 ± 0.9^{a,b}	3.5 ± 0.2^{a}	0.5 ± 0.0^{a}	1.2 ± 0.3^{a}	3.1 ± 0.2^{b}
22:6n-3	2.6 ± 0.5^{b}	3.7 ± 0.3^{b}	25.5 ± 1.7^{a}	2.3 ± 0.2^{a}	7.2 ± 3.9^{a}	24.5 ± 1.3^{b}
∑ Saturated	32.1 ± 1.9	32.6 ± 1.3	34.2 ± 0.5	37.5 ± 1.1	37.7 ± 0.7	39.1 ± 1.3
∑ Unsaturated						
∑ n-9	30.5 ± 1.6^{a}	9.4 ± 2.0^{b}	6.6 ± 0.9^{b}	34.0 ± 1.4^{a}	6.1 ± 0.5^{b}	6.5 ± 0.5^{b}
∑ n-6	30.0 ± 2.5^{b}	49.6 ± 0.8^{a}	26.3 ± 1.1^{b}	21.5 ± 0.4^{a}	43.9 ± 4.5^{b}	23.5 ± 0.4^{a}
∑ n-3	2.9 ± 0.5^{b}	5.4 ± 0.9^{b}	30.1 ± 1.6^{a}	3.7 ± 0.3^{a}	10.2 ± 4.1^{a}	28.7 ± 1.7^{b}
n-3/n-6	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	1.2 ± 0.1^{a}	0.2 ± 0.0^{a}	0.2 ± 0.1^{a}	1.2 ± 0.9^{b}

[a]—, Not detected; phospholipid fatty acid composition of soleus and extensor digitorum longus muscles after 9 wk on the three test diets, High n-6, EFAD, and High n-3. Only fatty acids detected at levels greater than 0.1 g/100 g of total fatty acids are listed.
[b]Values are means ± SEM, $n = 4$. The statistical significance of variation in mean phospholipid fatty acid levels among dietary treatments was assessed by one-way analysis of variance. Significantly different treatment means are denoted by different superscript roman letters. Abbreviation: EFAD, essential fatty acid-deficient.

High n-3 diet ran only 56% as long as those on the High n-6 diet ($P < 0.02$). The endurance of the EFAD group was also relatively low, being only 73% of that of the High n-6 group, although this difference was nonsignificant. Similarly, the amount of work done was significantly lower in both the High n-3 and EFAD groups [46 and 52% of the work performed by the High n-6 group ($P < 0.005$), respectively]. Very similar relationships persisted following recovery, where the High n-3 group ran for only 50% of the High n-6 group's mean endurance time ($P < 0.005$), and the High n-3 and EFAD rats did only 25 and 42% as much work as the High n-6 group ($P < 0.01$), respectively. Mean endurance time decreased by 49–55% for the three groups during the recovery period, and mean amount of work performed decreased by 73–85%.

We detected several litter effects throughout this study. For example, there were significant litter effects on endurance both after 9 wk on the test diet ($P < 0.02$) and after the 6 wk recovery period ($P < 0.05$). Although several rats needed encouragement to run, there were few that refused. Those that did were not included in the dataset. One rat that refused to run at 9 wk also refused following recovery. Also, his two littermates, which ran well after the 9-wk test diet, refused to run after the 6-wk recovery diet. These litter effects may represent real effects of variation among litters, or show day-to-day variation in experimental conditions. However, since all littermates were tested on the same day, and litter was one of the two factors considered in the ANOVA, these effects do not "interfere" with the description of the effects of dietary treatments.

DISCUSSION

Our study demonstrates that manipulation of the composition of dietary fatty acids can affect whole-animal physical performance in rats. We found three important effects: (i) that treadmill endurance (both "time to exhaustion" and "work performed") was influenced by the relative composition of n-6 and n-3 PUFA in the diet, (ii) that this effect may not be easily reversed, in that the differences between dietary groups persisted even after all rats were fed a common stock diet for 6 wk after being fed the test diets, and (iii) that treadmill endurance decreased in all groups when they were fed the same common diet after the experimental period.

Endurance and work were both dramatically reduced by consumption of the High n-3 diet, compared with those fed the High n-6 diet. Rats fed the High n-3 diet ran for only 56% as long as the High n-6 rats. These diets had the same total fat content, but the n-6 PUFA content in the High n-6 diet was partly replaced with n-3 fatty acids in the High n-3 diet. To our knowledge, this is the first study to report a substantial influence of the type of dietary polyunsaturated fat on whole-animal performance. These results were part of a larger study examining the role of dietary polyunsaturates (using the same three diets) on (i) composition of muscle phospholipids (13)

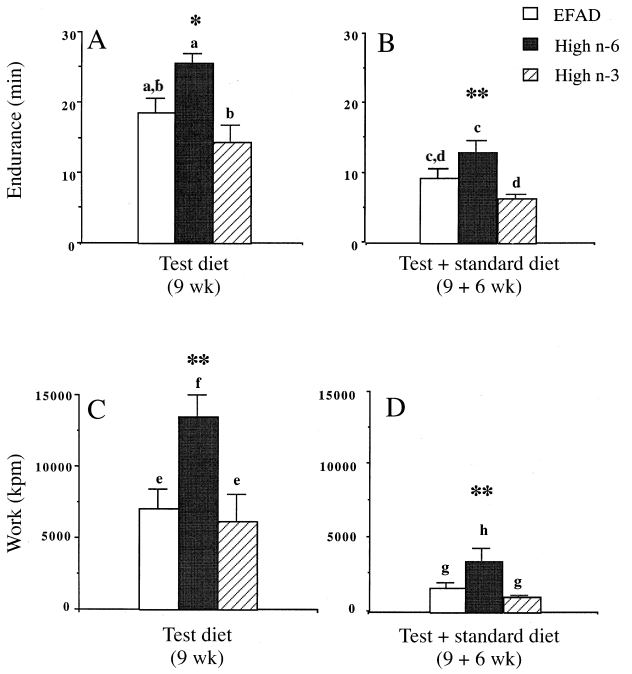

FIG. 1. The influence of diets of differing fatty acid composition on the submaximal treadmill endurance of rats. Figures A and B show the effect on endurance time whereas Figures C and D show the effect on work performed. Figures A and C show the effect after 9 wk on the test diets, and Figures B and D show the results obtained after a further 6 wk on a stock laboratory diet for all groups. Columns with different letters are significantly different from each other. $^{*}P < 0.05$; $^{**}P < 0.01$. Abbreviations: EFAD, essential fatty acid-deficient; High n-6, diet high in n-6 fatty acids; High n-3, diet high in n-3 fatty acids.

and (ii) contractile properties of isolated muscles (14), and whole-animal performance in rats. Recently, we have also shown that a diet rich in saturated fats is detrimental to endurance performance of rats when compared to diets either low in fat (i.e., carbohydrate-rich) or containing mainly n-6 polyunsaturated fats (12).

Whole-animal endurance during treadmill running is possibly influenced by a wide and complex range of physiological parameters. For example, limiting factors may sometimes occur in the respiratory and/or cardiovascular systems rather than the muscles themselves and similarly, the role of motivational differences is not easy to determine. In this study, we were particularly careful in trying to negate motivational influences between experimental groups by concealing the dietary treatment of each rat from the tester.

We have no direct knowledge of the possible mechanistic basis of the effects we have observed, but our previous studies can give some insight. The fact that the current dietary manipulations do not have any substantial influence on the *in vitro* contractile properties (including time to fatigue) of either isolated fast or slow muscles of rats (14) implies that the current dietary effects are not related to changes in the contractile properties of the muscles *per se*.

Limitation in substrate supply is a potential mechanism relating the type of dietary fat to treadmill endurance. A recent examination of the substrate and oxygen pathways during exercise at 40% maximal rate of oxygen consumption ($\dot{V}O_2$) max in both the highly aerobic dog and less aerobic goat has shown that about 70% of the energy requirements are provided from fat, and that at 60% $\dot{V}O_2$ max, its relative contribution is decreased to approximately half of total energy turnover (18). About 65–70% of the fat metabolized in these cases is from intramuscular triglyceride stores, which illustrates the relative importance of these stores during submaximal exercise. Whether the current diets resulted in different intramuscular triglyceride reserves in the rat is unknown. It is of interest that dietary n-3 fats increase the insulin sensitivity of muscle in rats (19). In humans, there is an inverse relationship between muscle insulin sensitivity and muscle triglyceride content (20). If this is also the case in rats, then the difference in treadmill endurance between rats fed the High n-6 and High n-3 diets may in turn be related to differences in intramuscular triglyceride stores. This possibility requires further investigation.

Dietary n-3 fats restore tissue insulin sensitivity in rats that are insulin-resistant, and thus they increase glucose entry and utilization in rat muscles (21). However, such effects may not be important in explaining the current results since oxidation of circulating glucose is only responsible for about 10–20% of total aerobic metabolism during treadmill exercise of dogs and goats (22). If, however, insulin-regulated glucose entry into muscle is important in determining treadmill endurance of rats, then we would expect that dietary n-3 enrichment would result in an increased endurance whereas we observed the opposite effect, a decrease in treadmill endurance.

Different types of fatty acids are metabolized *in vivo* at different rates. In rats, n-3 linolenic acid is metabolized more rapidly than n-6 linoleic acid (11). Furthermore, the rate of oxidation of n-3 linolenic acid is known to be influenced by the nature of the other fats in the diet, with linolenic acid being metabolized most rapidly when the other dietary fats are saturated and slowest when the other fats are predominantly n-6 polyunsaturates (19). Whether such differences are related to the endurance difference observed in the current study is unknown.

Dietary n-3 fats have been shown to have substantial influences on cardiovascular function and most of these are regarded as beneficial (23). If reduced cardiovascular function during exercise is the mechanism behind our dietary effect, then increased dietary intake of n-3 fats may not be so beneficial as generally thought. Using the same treadmill protocol, Baldwin *et al*. (15) showed that rats with (drug-induced) enlarged hearts have only a slightly greater endurance ($P < 0.1$) than normal rats.

Both n-6 and n-3 fatty acids are intimately involved in many aspects of physiological regulation in that they are both important membrane constituents and are also precursors of the eicosanoids, which are important chemical messengers (24). Whether the different diets resulted in different levels of eicosanoid production is unknown, but it is possible that the effects we report here are the result of such changes in eicosanoid messenger levels.

The second important finding of this study was that, although all rats were transferred to a common stock diet for 6 wk following the 9-wk test diet period, the relative differences between the experimental groups remained. This may indicate that the diet-induced differences are long-term and at least partly irreversible. Using the same diets, we have shown that in both slow (soleus) and fast (EDL) muscles the relative n-3 and n-6 composition of muscle phospholipids is related to their relative dietary presence (13; and see Table 1) and that even after 6 wk on the common diet the relative n-3 and n-6 composition of muscle phospholipids was little changed from what it was on the test diets. As we reported previously (13), the levels of n-3 fatty acids in the phospholipids of both soleus and EDL muscles were still significantly higher in rats from the High n-3 group than the other two groups after 6-wk recovery. This may be related to the persistent decrease in endurance seen in these rats after the recovery period.

All groups showed the same relative decrease in endurance when transferred to the common stock diet. Since all rats were 6 wk older and 50–60 g heavier, it may be that general aging was the cause. In a separate group of rats fed the same diets, there were no significant decreases in muscle mass or length following the common diet (14). This suggests that muscle atrophy is not an important component of the decline in treadmill endurance. Another explanation could be that the test diets were all 22E% fat, whereas the stock diet contained only 12E% fat. A number of studies have shown that an increase in the fat content of the diet can result in improved exercise performance in rats (2–4), dogs (5), and humans (6,7). Thus, it may be that a decrease in the fat content of the stock diet, compared to all three test diets, is the common factor that explains the decreased endurance in all groups. However, the important point is that the pattern of results was almost identical to the results after 9 wk on the test diets.

We have shown that rats fed diets with the same fat content but different lipid compositions show different endurances during submaximal running on a treadmill. The most surprising result was that the balance of n-3 and n-6 polyunsaturated in the diet has a highly significant influence on exercise performance of rats. We have used sedentary rats in the current study. Whether the same is true for trained rats or for humans is yet to be determined. Similarly, knowledge of the underlying mechanisms awaits further research.

In general, recommendations for weight reduction and amelioration of coronary artery disease have lead to increased consumption of PUFA containing high levels of n-6 fatty acids (25) and thus an increase in the dietary n-6/n-3 ratio. However, since the n-3 PUFA have been associated with decreased coronary artery disease, decreasing the dietary n-6/n-3 ratio will reduce the risks from deleterious eicosanoid production. Owing to publicity surrounding the findings that n-3 fatty acids are beneficial for the cardiovascular system and neural development, there has been an increase in human consumption of foods such as canola-based products and fish. Clearly however, the side effects of such increased consumption on physical performance in humans need to be investigated.

In conclusion, dietary fatty acids clearly exert significant effects on whole-animal performance. What determines submaximal treadmill running endurance is not known and difficult to ascertain. Effects on whole animals may reflect changes in muscles themselves or in the levels of eicosanoids, which are known to exert effects on secretory, cardiovascular, and immune functions (24). At present, diets containing a high level of PUFA relative to saturated fatty acids (and particularly the n-3 fatty acids) are recommended owing to their obvious benefits for the cardiovascular system (26). However, our results show a striking difference in effect for two diets that differ only in their ratio of the n-6 and n-3 fatty acids and point to the need for further research on the effects of such diets.

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The Fatty Acid Composition of a *Vibrio alginolyticus* Associated with the Alga *Cladophora coelothrix*. Identification of the Novel 9-Methyl-10-hexadecenoic Acid

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ABSTRACT: The fatty acid composition of a new strain of *Vibrio alginolyticus*, found in the alga *Cladophora coelothrix*, was studied. Among 38 different fatty acids, a new fatty acid, 9-methyl-10-hexadecenoic acid and the unusual 11-methyl-12-octadecenoic acid, were identified. Linear alkylbenzene fatty acids, such as 10-phenyldecanoic acid, 12-phenyldodecanoic acid and 14-phenyltetradecanoic acid, were also found in *V. alginolyticus*. The alga contained 43% saturated fatty acids, and 28% C_{16}–C_{20} polyunsaturated fatty acids of the n-3 and n-6 families.

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The association of marine bacteria with their invertebrate hosts is quite intriguing and of importance in marine biotechnology despite the fact that true symbiosis is difficult to assess (1). Most marine bacteria are found in heterogeneous aggregations, mostly adhered to inanimate surfaces. These surface interactions are dynamic and important in bacterial reproduction and survival (1). For example, marine algae are often found to be associated with a large variety of bacterial strains (2).

Despite the fact that much is known about the fatty acid composition of marine bacteria, recent findings indicate that there are still many unrecognized bacterial strains, either in seawater or associated with marine invertebrates, with unprecedented and/or unusual fatty acids that have not been fully studied or characterized (3). In the present study we report the fatty acid composition of a *Vibrio alginolyticus* and a Gram-positive bacillus associated with the alga *Cladophora coelothrix* from the Black Sea. Although these bacterial strains may not be widely distributed in association with the alga, they do contain several unusual fatty acids, in particular the previously unreported 9-methyl-10-hexadecenoic acid (**1**), the unusual 11-methyl-12-octadecenoic acid (4), and linear alkylbenzene fatty acids.

Algae from the genus *Cladophora* (Chlorophyta, Siphonophyceae, Cladophoraceae) are found worldwide, and some of these algae have the ability to survive up to 100% salinity (5). Only a few algae can survive such a change in salinity as the *Cladophora* (5). The principal fatty acids in the Chlorophyta are C_{16} and C_{18} polyunsaturated fatty acids such as 16:4n-3 and 18:3n-3 (6). The fatty acid composition of *C. coelothrix* has not been reported before.

MATERIALS AND METHODS

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

Collection. Cladophora coelothrix was collected in September 1994 at a beach near the village of Ravda, at the southern part of the Bulgarian Black Sea coast. The samples were covered by seawater and transported the same day to Sofia. Water samples were also collected at the same site. A part of the alga was also acclimated in an aquarium with seawater for one year in order to determine which original marine bacteria can survive under these conditions. The seawater parameters were kept constant with addition of artificial seawater (Provasoli; Sigma Chemical Co., St. Louis, MO).

Bacterial isolation. Cladophora coelothrix was cut into small pieces and ground in a porcelain mortar. The homogenates obtained were diluted in standard nutrient broth with a final salt concentration of 16‰ (equal to the concentration of salts in the Black Sea). The following dilutions were used: 10^{-1} (0.1 mL water or homogenate + 0.9 mL

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Abbreviations: ECL, equivalent chainlength; GC/MS, gas chromatography/mass spectrometry; SAM, *S*-adenosyl methionine.

broth), 10^{-2}, 10^{-3}, and 10^{-4}. From every dilution, 0.1 mL were taken and inoculated into two Petri dishes containing standard nutrient agar with a final concentration of 16‰ NaCl. After incubation at 37°C for 24 h, the morphology of the material obtained was established. The bacterial isolates were repeatedly streaked to fresh nutrient agar plates in order to ensure that they were in pure culture. In most cases a single cultivation was enough. Each strain was transferred to separate flasks and cultivated in standard nutrient broth, with aeration at 37°C, in order to obtain enough quantity for chemical analyses. We used 37°C because: (i) at this temperature, pathogenic bacteria are easily isolated, and (ii) the production of bacterial biomass is intensified. No qualitative differences were observed in our bacterial composition in a temperature range of 20–45°C. The bacterial biomass thus obtained, from every strain, was separated by centrifugation of the cultured liquid (6000 rpm for 20 min), and the lipids were extracted as described by Christie *et al.* (7).

Bacterial identification. Isolated bacterial strains were identified as *V. alginolyticus* (MB-1) and Gram-positive bacillus (OB-5 as well as other strains labeled by us as AB-1 and AB-2) (8–10). Characterization was done by routine biochemical and antibiotic tests modified for marine bacteria. The API 20E system (Analytab Products, Plainview, NY) was also used for characterization, as recommended by MacDonell *et al.* (11) for marine isolates.

Fatty acid isolation. The lipids from *C. coelothrix*, and from the associated bacteria, were extracted with chloroform/methanol (2:1, vol/vol) and saponified. The nonsaponifiables were removed, and the acids were methylated with 1% sulfuric acid in methanol (7).

Derivatives. The methyl esters were hydrogenated in 10 mL of absolute methanol and catalytic amounts of platinum oxide (PtO_2). The double-bond positions in the monoenoic fatty acid methyl esters were determined by dimethyl disulfide derivatization (12). Pyrrolidide derivatives were also prepared as previously described (13). Mass spectral data for methyl 9-methyl-10-hexadecenoate and methyl 11-methyl-12-octadecenoate, as well as their corresponding dimethyl disulfide derivatives, follows.

Methyl 9-methyl-10-hexadecenoate. MS *m/z* (relative intensity) M^+ 282(1), 251(2), 239(1), 227(1), 199(1), 183(2), 166(4), 143(11), 140(5), 111(9), 87(25), 83(33), 81(15), 74(43), 69(63), 67(21), 59(20), 57(23), 55(100).

Methyl 9-methyl-10,11-bis(methylthio)hexadecanoate. MS *m/z* (relative intensity) M^+ 376(3), 346(1), 299(3), 281(1), 245(8), 213(3), 197(6), 185(3), 165(9), 147(7), 131(15), 99(8), 97(14), 95(14), 87(16), 83(28), 81(23), 74(22), 71(29), 69(44), 67(25), 61(34), 59(21), 57(65), 55(100).

Methyl 11-methyl-12-octadecenoate. MS *m/z* (relative intensity) M^+ 310(1), 279(2), 211(2), 194(3), 179(2), 171(5), 152(1), 140(7), 138(3), 133(1), 125(7), 123(3), 111(6), 109(4), 97(14), 95(9), 87(11), 81(14), 74(23), 69(65), 67(22), 57(22), 55(100).

Methyl 11-methyl-12,13-bis(methylthio)octadecanoate. MS *m/z* (relative intensity) M^+ 404(3), 373(1), 310(1), 274(4), 273(21), 241(8), 171(3), 169(1), 157(2), 131(29), 109(10), 97(16), 95(21), 87(18), 83(38), 81(26), 74(32), 69(46), 67(26), 61(51), 55(100).

RESULTS

Fatty acid composition of C. coelothrix. The fatty acid composition of *C. coelothrix,* as well as that of associated microorganisms, is presented in Table 1. In contrast to other Chlorophyta, such as *Cladophora stimpsonii*, significant amounts of 16:3n-6 and 18:3n-6 were found in *C. coelothrix* (6). Hexadecanoic acid (16:0) was the most abundant (28% of the total) fatty acid. The typical bacterial *iso/anteiso* fatty acids with chainlengths between C_{15}–C_{17} were also identified in *C. coelothrix.*

Fatty acid composition of V. alginolyticus. Several associated bacterial strains were isolated from *C. coelothrix*, probably as epiphytes. Gram-positive bacilli and a new strain of *V. alginolyticus* were identified as described in the Materials and Methods section. The new strain of *V. alginolyticus* was only detected in *C. coelothrix*, and it was not found in the surrounding seawater. The fatty acid composition of *V. alginolyticus* is also presented in Table 1. The principal fatty acids identified in *V. alginolyticus* correspond to those previously reported for other *Vibrio* (14). However, 38 different fatty acids were identified in this *V. alginolyticus*, in contrast to only 17 fatty acids that were previously reported in other *Vibrio* strains (14). Of the 38 fatty acids identified in *V. alginolyticus*, 34% were saturated, 44% monounsaturated, 15% branched *iso/anteiso*, and 7% were linear alkylbenzene fatty acids (Table 1). The monounsaturated fatty acid profile of this bacterium was complex, but in general Δ9 desaturation predominated. Most of the monounsaturated fatty acids had the *cis* stereochemistry, but two *trans* fatty acids were also identified by their equivalent chainlength (ECL) values and corresponding mass spectra of their methyl esters. These acids were (*E*)-9-hexadecenoic acid and (*E*)-11-octadecenoic acid, the latter probably arising from a two-carbon extension of the former.

Two unusual methyl-branched fatty acids were identified in *V. alginolyticus*. These were characterized as 9-methyl-10-hexadecenoic acid (**1**) and 11-methyl-12-octadecenoic acid, which were characterized as methyl esters. To the best of our knowledge, **1** is novel and 11-methyl-12-octadecenoic acid has only been detected before in *Mycobacterium fallax* (4). Methyl 9-methyl-10-hexadecenoate presented an ECL value of 16.03, while methyl 11-methyl-12-octadecenoate presented an ECL value of 18.06 in capillary GC, indicating monounsaturation and methyl branching in the middle of the chain. The mass spectra of the methyl ester of **1** and its longer chain analog displayed molecular ion peaks at M^+ 282 and M^+ 310, respectively, with their corresponding $M^+ - 32$ peaks at *m/z* 250 and *m/z* 279. Upon catalytic hydrogenation of the whole fatty acid methyl ester mixture, methyl 9-methylhexadecanoate (ECL = 16.37) and methyl 11-methyloctadecanoate (ECL = 18.39) were obtained as two of the products. The mass spectrum of methyl 9-methylhexadecanoate displayed a M^+ 284,

TABLE 1
The Fatty Acid Composition of the Alga *Cladophora coelothrix* and Associated Bacterial Strains

	Relative abundance (w/w)		
Fatty acids	*C. coelothrix*	MB-1[a]	OB-5[b]
10-Me-12:0	—	—	2.0
12:0	—	0.1	2.0
11-Me-13:0	—	0.3	11.7
10-Me-13:0	—	—	3.4
13:0	—	0.2	—
12-Me-14:0	—	0.4	7.5
7-14:1	—	0.3	—
14:0	8.7	1.3	8.0
13-Me-15:0	1.0	3.6	22.0
12-Me-15:0	0.5	0.2	9.1
7-15:1	—	0.6	—
9-15:1	—	0.5	—
15:0	1.2	8.4	2.3
4,7,10-16:3	5.4	—	—
4,7,10,13-16:4	5.2	—	—
14-Me-16:0	0.3	2.4	6.0
16:2	1.2	—	—
7-16:1	2.6	—	—
9-16:1	6.1	15.1	—
9*t*-16:1	—	4.4	—
11-16:1	—	0.4	—
16:0	28.7	12.6	14.2
9-Me-10-17:1	—	0.3	—
15-Me-9-17:1	—	0.5	—
15-Me-17:0	0.7	7.1	4.7
14-Me-17:0	0.3	0.1	3.3
9-17:1	—	7.7	—
11-17:1	—	3.1	—
17:0	0.7	9.4	1.1
10-Ph-10:0	—	0.9	—
16-Me-18:0	—	0.7	—
6,9,12-18:3	5.7	—	—
6,9,12,15-18:4	0.3	—	—
9,12-18:2	7.8	—	—
9-18:1	10.5	0.3	—
11-18:1	5.7	9.1	—
11*t*-18:1	—	0.3	—
13-18:1	—	0.1	—
18:0	3.3	2.0	1.6
11-Me-12-19:1	—	0.6	—
17-Me-19:0	—	0.3	0.1
11-19:1	—	0.3	—
13-19:1	—	0.1	—
12-Ph-12:0	—	4.5	—
5,8,11,14-20:4	2.3	—	—
5,8,11,14,17-20:5	1.0	—	—
13-20:1	0.2	0.3	—
20:0	0.2	—	—
14-Ph-14:0	—	1.5	—
16-Ph-16:0	—	0.1	—

[a]The MB-1 bacteria was identified as *Vibrio alginolyticus*. It was only detected in the alga.
[b]The OB-5 bacteria was identified as a Gram-positive bacillus. It was initially detected in both the seawater and the alga. Other similar strains, with similar fatty acid composition, were isolated from the alga and not the water when the alga was cultivated in an aquarium with artificial seawater.

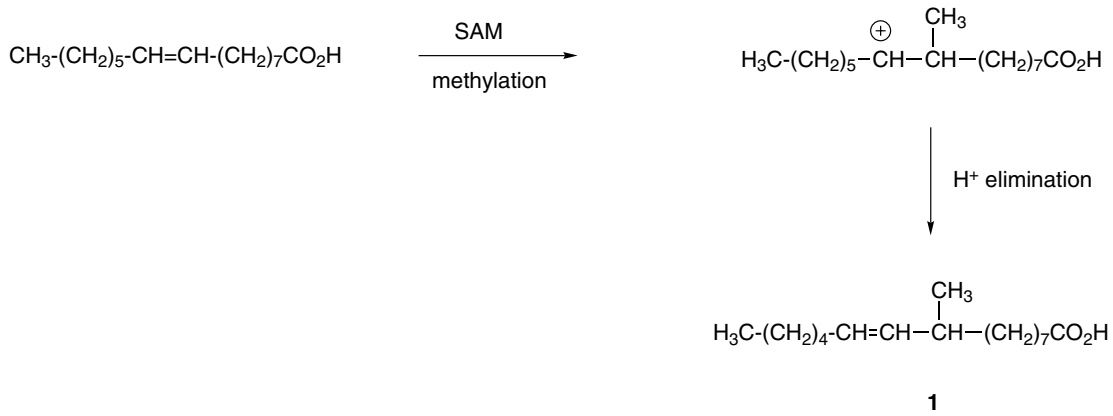
and strong fragmentations at *m/z* 157 (7%) and *m/z* 185 (14%), together with a diminished peak at *m/z* 171 (2%), indicative of methyl substitution at C-9. On the other hand, the mass spectrum of methyl 11-methyloctadecanoate displayed a M^+ 312, and strong fragmentations at *m/z* 185 (11%) and *m/z* 213 (8%), together with a diminished peak at *m/z* 199 (1%), indicative of methyl substitution at C-11. Once the methyl substitutions were defined, the double-bond positions were determined by dimethyldisulfide derivatization (12). Upon reaction of the whole fatty acid methyl ester mixture with dimethyl disulfide and I_2, methyl 9-methyl-10,11-bis(methylthio)hexadecanoate and methyl 11-methyl-12,13-bis(methylthio)octadecanoate were obtained as two of the products. The mass spectra of these derivatives presented molecular ion peaks at M^+ 376 and M^+ 404. The key fragmentations in methyl 9-methyl-10,11-bis(methylthio)hexadecanoate were observed at *m/z* 131 (15%) and *m/z* 245 (8%). On the other hand, the key fragmentations in methyl 11-methyl-12,13-bis(methylthio)octadecanoate were observed at *m/z* 131 (29%) and *m/z* 273 (21%), indicative of double-bond substitution at C-12. All of the above mass spectral data confirm methyl 9-methyl-10-hexadecenoate and methyl 11-methyl-12-octadecenoate as the most probable structures.

An interesting finding in *V. alginolyticus* was the identification of the linear alkylbenzene fatty acids 10-phenyldecanoic acid, 12-phenyldodecanoic acid, 14-phenyltetradecanoic acid, and 16-phenylhexadecanoic acid. These linear alkylbenzene fatty acids were identified, as methyl esters, by MS comparing to reported mass spectral data (National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health mass spectral library from the U.S. Department of Commerce). The mass spectra of these methyl esters were all similar, i.e., a base peak at *m/z* 91, and abundant fragmentations at *m/z* 55, 74, 104, and a strong $M^+ - 32$ peak. The straight alkyl chain was further confirmed by pyrrolidine derivatization, since the mass spectra of the pyrrolidide derivatives presented a continuous series of ion clusters, 14 amu apart, starting at the carbonyl end of the chain and finishing at the penultimate carbon before the benzene ring, where a gap of 91 amu was observed before the molecular ion peak (13). Moreover, a linear plot was observed when the capillary GC retention times of these C_{10}–C_{16} methyl phenylalkanoates were plotted against carbon chainlength.

Fatty acid composition of an associated Gram-positive bacillus. Several associated Gram-positive bacilli were found in *C. coelothrix*. The fatty acid composition of one of them, namely OB-5, is presented in Table 1. This bacillus was initially detected in both the seawater and the alga, and therefore it seems to be not exclusive of the alga. This Gram-positive bacillus presented a complete saturated fatty acid profile. In particular, branched *iso*/*anteiso* fatty acids with chainlengths between C_{13}–C_{17} were the most abundant. No monounsaturated fatty acids were detected.

DISCUSSION

Our findings with *V. alginolyticus* and *C. coelothrix* present new perspectives in terms of the fatty acid composition of microorganisms associated with algae. The identification of so



1

SCHEME 1

many different unusual fatty acids in a single *Vibrio* species is rare (14). The characterization of the novel 9-methyl-10-hexadecenoic acid (**1**) is also of interest. Its longer-chain analog 11-methyl-12-octadecenoic acid has only been reported before from *Mycobacterium fallax* (4). In *M. fallax* it was shown, by feeding [methyl-$^2H_3$]L-methionine, that 11-methyl-12-octadecenoic acid originated from 11-octadecenoic acid by a process of *S*-adenosyl methionine (SAM) methylation followed by hydrogen elimination (4). In our *V. alginolyticus,* 9-methyl-10-hexadecenoic acid (**1**) may have originated from 9-hexadecenoic acid, which was particularly abundant in this *V. alginolyticus*, by a similar process of SAM methylation followed by hydrogen elimination (Scheme 1).

A comparison of the fatty acid composition of the associated bacterial strains with that of the alga *C. coelothrix* reveals some common fatty acids. For example, saturated fatty acids between C_{14}–C_{18} are common in all of the studied species (Table 1). The branched *iso*/*anteiso* fatty acids with chainlengths between C_{15}–C_{17}, which were also found in *C. coelothrix*, clearly have a bacterial origin. The monounsaturated fatty acids 9-hexadecenoic acid and 11-octadecenoic acid were also found in *C. coelothrix* and in *V. alginolyticus* (Table 1). The fact that this *V. alginolyticus* was only detected in the alga and not in the surrounding seawater is indicative that it could be a symbiont.

The identification of linear C_{10}–C_{16} alkylbenzene fatty acids in our strain of *V. alginolyticus* is also of interest. These alkylbenzene fatty acids could have originated from linear alkylbenzene hydrocarbons present in the sea. Linear alkylbenzenes are starting materials for the industrial manufacturing of linear alkylbenzene sulfonate surfactants *via* the process of sulfonation (15). In most cases the sulfonation process, although efficient, does not go to completion, and unreacted linear alkylbenzenes, typically between C_{10}–C_{14} carbons, are carried into detergents. Some marine bacteria, such as *Alcaligenes* sp. and *Acinetobacter* sp., have been shown to degrade alkylbenzenes in high yields (16). The biodegradation process follows a classical mechanism, i.e., oxidation at the terminal methyl end of the chain followed by a series of β-oxidation steps until final conversion to benzoic acid (16). Nevertheless, there is still the possibility that the alkylbenzene fatty acids could have arisen from *de novo* biosynthesis from benzoic acid. Work is in progress in our laboratories elucidating the fatty acid composition of marine bacteria and their invertebrate hosts.

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Production of 3*R*-Hydroxy-polyenoic Fatty Acids by the Yeast *Dipodascopsis uninucleata*

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ABSTRACT: Various fatty acids were fed to the yeast *Dipodascopsis uninucleata* UOFS Y 128, and the extracted samples were analyzed for the accumulation of 3-hydroxy metabolites with the help of electron impact gas chromatography–mass spectrometry. Fatty acids containing a 5*Z*,8*Z*-diene system (5*Z*,8*Z*,11*Z*-eicosatrienoic, 5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic, and 5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acids) yielded the corresponding 3-hydroxy-*all*-*Z*-eicosapolyenoic acids. Moreover, linoleic acid (9*Z*,12*Z*-octadecadienoic acid) and 11*Z*,14*Z*,17*Z*-eicosatrienoic acid were converted to the 3-hydroxylated metabolites of shorter chain length, e.g., 3-hydroxy-5*Z*,8*Z*-tetradecadienoic acid and 3-hydroxy-5*Z*,8*Z*,11*Z*-tetradecatrienoic acid, respectively. In contrast, no accumulation of a 3-hydroxy metabolite was observed with oleic acid (9*Z*-octadecenoic acid), linolelaidic acid (9*E*,12*E*-octadecadienoic acid), γ-linolenic acid (6*Z*,9*Z*,12*Z*-octadecatrienoic acid), and eicosanoic acid as substrate. These findings pinpoint that the 3-hydroxylation of a fatty acid in *Dipodascopsis uninucleata* requires a 5*Z*,8*Z*-diene system either directly or following initial incomplete β-oxidation. Following analysis of the enantiomer composition, the arachidonic acid metabolite was identified as 3*R*-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid, which rules out a normal β-oxidation as biosynthetic route to this new class of oxylipins.

Lipids 32, 1277–1283 (1997).

Long-chain hydroxy fatty acids and other oxygenated fatty acid derivatives (oxylipins) are widely distributed in both the animal and plant kingdom and exert a plethora of biological actions (1–3). During the past 10 yr, plenty of knowledge has accumulated regarding the synthesis of oxylipins by fungi and related organisms. A large number of unsaturated hydroxy fatty acids which are formed by either lipoxygenase, dioxygenase, or cytochrome P-450-mediated pathways have been found in various fungal species and oomycetes (4–11). These compounds carry one or more hydroxy groups at carbon atoms 5, 7, 8, 9, 12, 13, 15, or 17 of the fatty acid molecule. While the majority of fungal oxylipins are formed from oleic or linoleic acid, there are only a few examples for arachidonic acid (AA)-derived oxylipins (eicosanoids) in fungi. The 15*S*-hydroxyeicosanoids have been identified in *Saprolegnia* and *Achlya* species (4,5). In earlier communications, we have reported on the formation of 3-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (3-HETE) by the yeast *Dipodascopsis uninucleata* fed with AA (12–13). In the present study, we demonstrate that 3-hydroxy fatty acids are produced by *D. uninucleata* from other polyenoic fatty acids, provided that they contain a 5*Z*,8*Z*-diene system either directly or upon preceding β-oxidation. Moreover, the stereochemical identification of the 3-hydroxy group (3*R*) rules out a normal β-oxidation as putative biosynthetic pathway for these oxylipins.

EXPERIMENTAL PROCEDURES

The compound 3-HETE was isolated and purified as described elsewhere (12). The methylation and trimethylsilylation were performed according to the instructions supplied with the kits from Aldrich (Deisenhofen, Germany) and Merck Chemicals (Darmstadt, Germany), respectively. Fatty acids 18:1 (9*Z*) and 18:2 (9*Z*:12*E*) were obtained from Sigma (Deisenhofen, Germany); all other fatty acids were supplied by Cayman (Ann Arbor, MI).

*Synthesis of 3*R- *and 3*S-*HETE.* The synthetic strategy for 3*R*- and 3*S*-HETE involved a convergent approach coupling chiral aldehyde with Wittig salt; these were derived from 2-deoxy-D-ribose and AA, respectively (14). Briefly, lead tetraacetate oxidation of readily available methyl 5,6-dihydroxyeicosatetraenoate (15) and $NaBH_4$ reduction of the resultant aldehyde gave an alcohol. Alcohol to bromide interconversion under standard conditions followed by displacement with triphenylphosphine in acetonitrile generated a phosphonium salt. The 3*R*-*t*-butyldiphenylsilyloxy *d*-valerolactone (16) prepared from 2-deoxy-D-ribose as described

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Abbreviations: AA, arachidonic acid; HETE, hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

(17) was converted to an ester *via* saponification and treatment with ethereal diazomethane, the oxidation of which with pyridinium chlorochromate yielded chiral aldehyde. The condensation of chiral aldehyde with the ylide of phosphonium salt gave, after fluoride-mediated deprotection and high-performance liquid chromatography (HPLC) purification, methyl 3*R*-hydroxy-5,8,11,14-eicosatetraenoate. Mitsunobu inversion of it using chloroacetic acid, saponification of the resultant ester, and diazomethane esterification yielded methyl 3*S*-hydroxy-5,8,11,14-eicosatetraenoate. The corresponding free acids were prepared by saponification of the respective esters.

Cultivation of the yeast D. uninucleata*. Dipodascopsis uninucleata* UOFS Y128 was grown to stationary phase (sexual stage) after which 12.5 mg/L of the fatty acid (Table 1) were added. Following 6 h of incubation, the cells were harvested. The growth and harvesting procedures were as described earlier (12).

Extraction of fatty acid metabolites from the yeast. The cells obtained in each experiment were mixed with absolute ethanol to a final concentration of 80% ethanol. The suspension was kept at 5°C for 18 h and then filtered. The filtrate was adjusted to 15% aqueous ethanol. The yeast sample was acidified to pH 3.0 with formic acid and chromatographed on a preconditioned Sep-Pak C_{18} cartridge (Millipore, Bradford, MA) as described (18). The AA metabolites were finally eluted with 5 mL of freshly distilled ethyl acetate. The eluate was evaporated under a stream of nitrogen, and the fatty acid metabolites were separated from other hydrophobic compounds by applying their triethylamine salts on Sep-Pak silica gel cartridges and eluting them with 15% ethanol. Following evaporation of ethanol under a stream of nitrogen, the samples were adjusted to pH 3.0 and extracted with ethyl acetate as described above. Each sample was again chromatographed on a preconditioned Sep-Pak C_{18} cartridge as described before and finally dissolved in chloroform.

Thin-layer chromatography (TLC). Chloroform fractions obtained from yeasts fed with the polyenoic fatty acid in the presence or absence of 1 mM acetylsalicylic acid were chromatographed together with the 3-HETE standard prepared in our own laboratory on silica gel plates (Merck) as described (12). The compounds were visualized by placing the TLC plates in an iodine tank.

Electron impact mass spectra. Electron impact mass spectra of 3-HETE and other 3-hydroxy fatty acids were recorded on a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) equipped with a Hewlett-Packard 5972 MSD at 1447 EM Volts. An HP-1 fused-silica capillary column (30 m × 0.25 mm i.d.) was used for separation with helium as carrier gas. Other operating conditions were: ion source 170°C and electron impact energy 70 eV.

Prior to analysis, the methyl–trimethylsilyl (ME–TMSi) derivatives of the samples were prepared as described (19) and reconstituted in 100 µL chloroform/hexane (1:4) before injection. Samples were injected by split ratio of 1:50 at 230°C and the column temperature programmed from 140–300°C at 5°C per min.

Analysis of enantiomeric composition of 3-HETE. Enantiomeric analysis of 3-HETE from *D. uninucleata* was performed on an Apex Chiral AU 50 HPLC column (4.6 × 250 mm; Jones Chromatography, Hengoed, Glamorgan, United Kingdom) with a variable wavelength ultraviolet-detector (Pharmacia, Freiburg, Germany) using 1% isopropanol in *n*-hexane as isocratic solvent system. The flow rate was 1.0 mL/min. The ultraviolet absorption peaks were monitored at 208 nm.

TABLE 1
Accumulation of 3-Hydroxy Metabolites from Selected Fatty Acids Fed to *Dipodascopsis uninucleata*

Fatty acid fed	Accumulation of oxylipin[a]	Identified structure
18:1 (9*Z*)	—	
18:2 (9*Z*,12*Z*)	+	3-OH-14:2 (5*Z*,8*Z*)
18:2 (9*Z*,12*E*)	—	
18:3 (6*Z*,9*Z*,12*Z*)	—	
20:0	—	
20:3 (5*Z*,8*Z*,11*Z*)	+	3-OH-20:3 (5*Z*,8*Z*,11*Z*)
20:3 (11*Z*,14*Z*,17*Z*)	+	3-OH-14:3 (5*Z*,8*Z*,11*Z*)
20:4 (5*Z*,8*Z*,11*Z*,14*Z*)	+	3-OH-20:4 (5*Z*,8*Z*,11*Z*,14*Z*)
20:5 (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)	+	3-OH-20:5 (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)

[a]As judged by the detection of a fraction comigrating with 3-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid in thin-layer chromatography.

RESULTS

A number of fatty acids were fed to *D. uninucleata.* After 6 h the fatty acids which were not incorporated in cellular lipids were extracted by ethanol and separated as described in the Experimental Procedures section. It is evident from Table 1 that a selected number of fatty acids produced various compounds which comigrated =in TLC with authentic 3-HETE. These compounds were isolated, converted to their ME–TMSi derivatives, and their structures were established by gas chromatography/electron impact mass spectrometry (Figs. 1–4). The characteristic fragments of the compounds are listed in Table 2. All compounds exhibited a base peak of *m/z* 175 [$CH_3O(CO)–CH_2–CHO–TMSi$] which is indicative of a hydroxylation at C-3 position. A 3-hydroxy derivative accumulated when 5*Z*,8*Z*,11*Z*-eicosatrienoic, 5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic (AA), 5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic, 11*Z*,14*Z*,17*Z*-eicosatrienoic, or 9*Z*,12*Z*-octadecadienoic (linoleic) acids were fed to the yeast. From the structural identification of the corresponding derivatives, it was evident that the first three fatty acids were 3-hydroxylated without alteration of the chain length, whereas the other two were shortened by six and four carbon atoms, respectively. In contrast, 3-hydroxy derivatives were not observed upon feeding of 9*Z*-octadecenoic (oleic), 9*E*,12*E*-octadecadienoic (linolelaidic), 6*Z*,9*Z*,12*Z*-octadecatrienoic (γ-linolenic), or eicosanoic acids, although these fatty acids were shown to be metabolized to a large extent by the yeast, most probably *via* β-oxidation. Feeding experiments with C_{22} polyenoic fatty acids failed

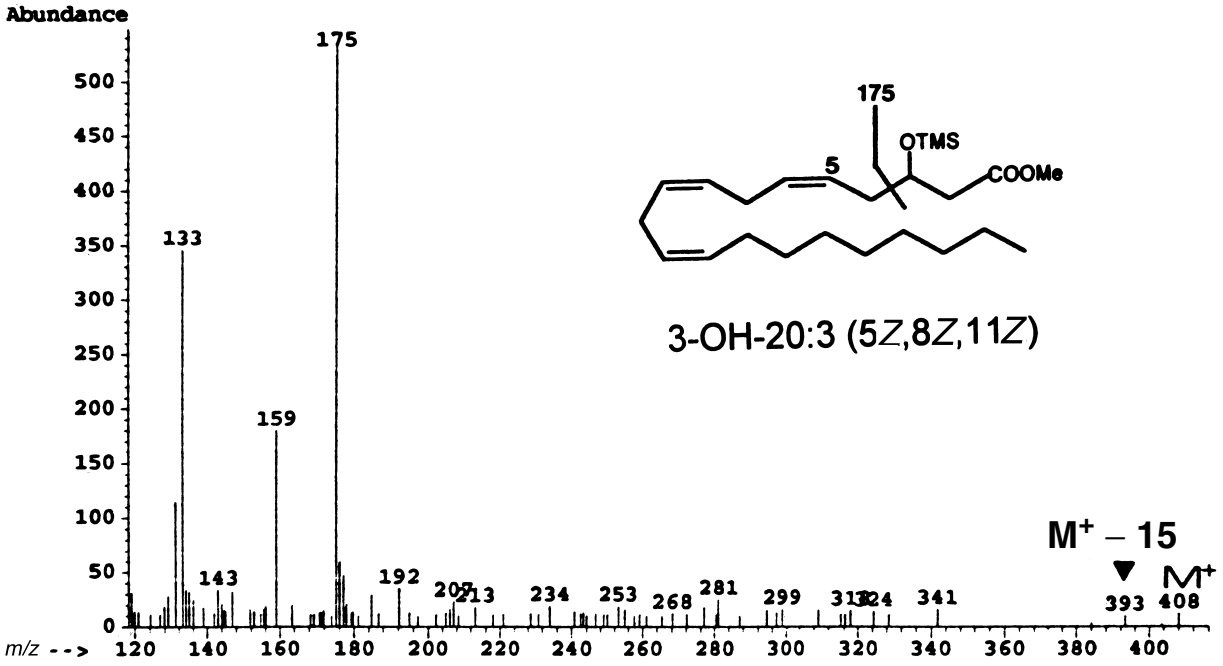

FIG. 1. Electron impact–mass spectra of methyl-trimethylsilylated 3-hydroxy 20:3 (5*Z*,8*Z*,11*Z*).

since the latter turned out to be toxic to *D. uninucleata*. The accumulation of the 3-hydroxy polyenoic fatty acids was generally suppressed by 1 mM acetylsalicylic acid.

For the product from AA, 3-HETE, an analysis of the enantiomeric composition was performed. Figure 5A shows the resolution of the enantiomers when injected in a ratio of 4:1 (40 µg 3*R*-HETE and 10 µg 3*S*-HETE). The retention volumes were 3.0 and 3.8 mL solvent, respectively. Figure 5B clearly shows that the natural 3-HETE from *D. uninucleata* consists of 95% 3*R*-HETE. Interfering peaks were not observed in the HPLC chromatogram at the above retention volumes (Fig. 5C). [In independent experiments these data were confirmed by Dr. M. Hamberg, Karolinska Institute, Stockholm, Sweden, who analyzed a sample of 3-HETE from *D. uninucleata* supplied by Dr. J. Friend, University of Hull, United Kingdom (personal communication).]

DISCUSSION

In this report we have demonstrated that *D. uninucleata* produced a 3-hydroxy derivative not only from AA but also from a variety of other polyenoic fatty acids, including linoleic acid which contributes to about one-fourth of the total fatty acids of this yeast (13). It is interesting to note that 3-HETE exhibited an enantiomeric ratio of 95:5 (*R/S*). On the basis of structural similarities between 3*R*-HETE and other 3-hydroxy fatty acids, we postulate that the other 3-hydroxy fatty acids should also bear the *R*-configuration. The observation that a number of other fatty acids, among them saturated fatty acids, oleic acid and γ-linolenic acid, were not converted to detectable amounts of 3-hydroxy derivatives, despite the fact that they were metabolized, suggests some specific structural requirements for the 3*R*-hydroxylation. From the comparison of the structures of the hydroxylation-susceptible and nonsusceptible fatty acids, and of those of the hydroxy derivatives, it follows that the presence of a 5*Z*,8*Z*-diene system in the fatty acid molecule is a prerequisite for 3*R*-hydroxylation by *D. uninucleata*. Such a diene arrangement can also be accomplished by a preceding partial breakdown of the fatty acid *via* β-oxidation, which explains the formation of 3-hydroxylated C_{14} polyenoic fatty acids from linoleic acid and 11*Z*,14*Z*,17*Z*-eicosatrienoic acid.

The 5*Z*,8*Z*-diene system in the fatty acid does not take part in the aforementioned biotransformation reaction, nor does it seem to be capable of activating the methylene group at the

TABLE 2
Characteristic Mass Fragments of the 3-Hydroxy Derivatives

Metabolite	Fragments	Figure
3-OH-20:3 (5*Z*,8*Z*,11*Z*)	175; 408 [M^+]; 393 [$M^+ - 15$ (CH_3)]	1
3-OH-20:4 (5*Z*,8*Z*,11*Z*,14*Z*)	175; 406 [M^+]; 391 [$M^+ - 15$ (CH_3)]	2A
3-OH-20:5 (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)	175; 404 [M^+]; 389 [$M^+ - 15$ (CH_3)]	2B
3-OH-14:2 (5*Z*,8*Z*)	175; 326 [M^+]; 311 [$M^+ - 15$ (CH_3)]	3
3-OH-14:3 (5*Z*,8*Z*,11*Z*)	175; 309 [$M^+ - 15$ (CH_3)]	4

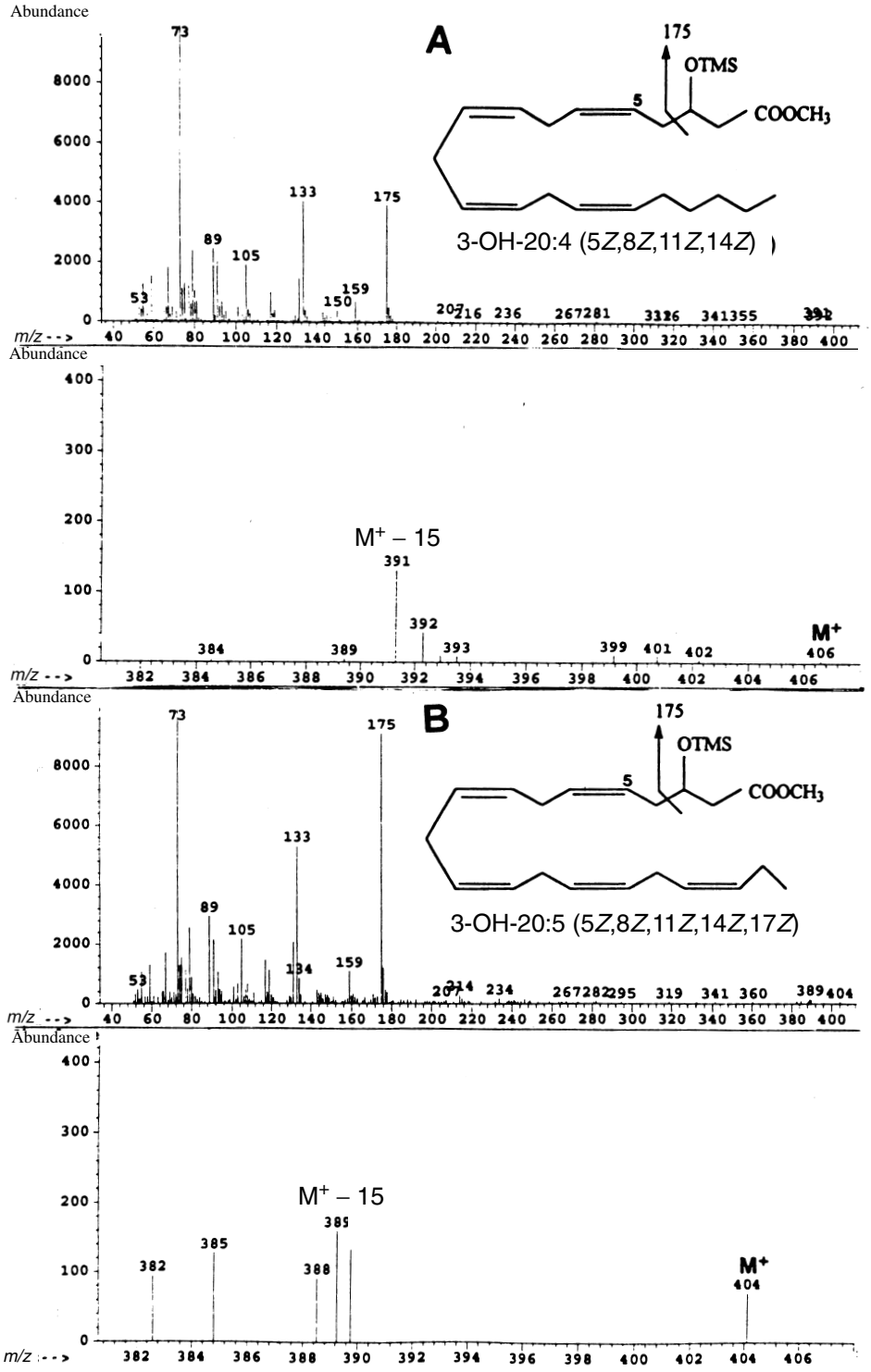

FIG. 2. Electron impact-mass spectra of methyl-trimethylsilylated 3-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (HETE) (A) and 3-HEPE (B). Lower panels show expanded spectra of *m/z* 380 to 408. OTMS, *O*-Trimethylsilyl. See Figure 1 for other abbreviation.

C-3 position at which hydroxylation occurs. Therefore, the 5*Z*,8*Z*-diene system might fulfill a signal function for the recognition of the substrate by the corresponding enzyme in *D. uninucleata via* binding at the active site.

At first sight, a 3-hydroxy fatty acid may be principally formed *via* incomplete β-oxidation. In that case, however, the 3*S* enantiomer (corresponding to 3-[L] according to the Fischer nomenclature) would have been found as the main metabolite. Since we clearly demonstrated that *D. uninucleata* produced nearly exclusively 3*R*-HETE (3-[D]-HETE)

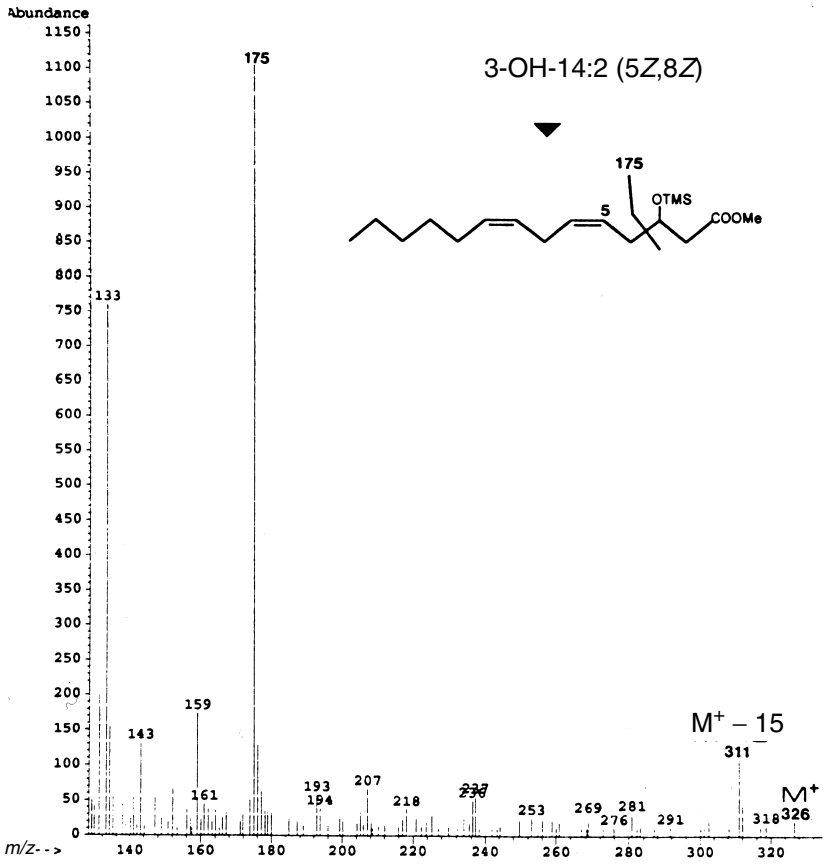

FIG. 3. Electron impact–mass spectra of methyl-trimethylsilylated 3-hydroxy 14:2 (5*Z*, 8*Z*). See Figure 1 for abbreviations.

from AA, such an assumption has to be rejected. The different stereospecificities of the biosynthetic pathway in *D. uninucleata* and β-oxidation further imply that the 3*R*-hydroxy fatty acids cannot be degraded *via* normal β-oxidation, which might be the reason for the accumulation of these oxylipins upon feeding of the corresponding precursor fatty acids to the yeast.

The capability of producing 3*R*-hydroxy fatty acids is apparently not restricted to *D. uninucleata* alone. *The* 3*R*-hydroxypalmitic acid and related saturated compounds have been reported in other yeasts such as *Rhodotorula graminis, Rh. glutinis* (20), and *Saccharomycopsis malanga* (21). Moreover, medium-chain 3-hydroxy fatty acids have been shown to be present in the glycolipids of the smut fungi *Ustilago zeae* and *U. nuda* (22). Finally, saturated 3*R*-hydroxy fatty acids with 10, 12, and 14 carbons are encountered in Lipid A, the component of the lipopolysaccharides of Gram-negative bacteria that is essential for endotoxin activity (23). The biosynthetic system in *D. uninucleata* appears to differ, however, from those of other fungi and of bacteria in its marked preference for polyunsaturated fatty acids possessing a 5*Z*,8*Z*-diene system.

The biosynthetic pathway to the 3*R*-hydroxy polyenoic fatty acids remains to be elucidated. Lipoxygenase- or dioxygenase-mediated reactions can be excluded since they would require a neighboring double-bond system (at C-2 and/or C-4). The same is true for the involvement of prostaglandin endoperoxide synthase, even though the synthesis of the 3*R*-hydroxy fatty acids was found to be inhibited by acetylsalicylic acid (12). It should be stressed that the action of acetylsalicylic acid on enzymes is fairly unspecific. Moreover, acetylsalicylate and salicylate act in higher plants as hormones inducing certain hypersensitivity reactions by changing the gene expression program (24,25). Thus, two metabolic routes to 3*R*-hydroxy fatty acids are conceivable: (i) a reaction sequence analogous to β-oxidation, however, implicating a 2-enoyl-CoA hydratase with opposite steric specificity; and (ii) a direct monooxygenase reaction at C-3, e.g., by a cytochrome P-450 type enzyme.

The conversion of linoleic acid to 3*R*-hydroxy-5*Z*,8*Z*-tetradecadienoic acid needs special attention in view of the fact that the precursor fatty acid contributes to about one-fourth of the total fatty acid content of *D. uninucleata* (13). Therefore, this oxylipin could exert a regulatory function during the sexual phase of the reproductive cycle of this yeast as has been shown to be the case for other fungal oxylipins (9).

Since *D. uninucleata* does not synthesize adequate amounts of AA, the conversion of the latter to 3*R*-HETE does not appear to be of major importance for the reproduction biology of this yeast. This reaction may, however, play a pivotal role in the cell-cell interaction with AA-producing organisms. In separate investigations we found that 3*R*-HETE af-

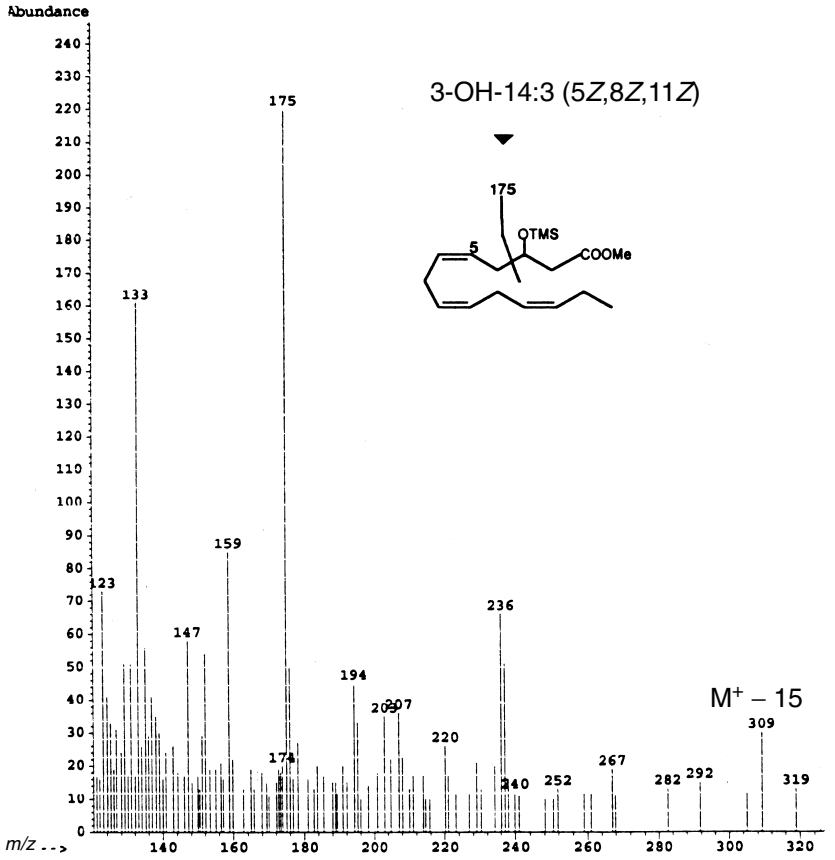

FIG. 4. Electron impact–mass spectra of methyl-trimethylsilylated 3-hydroxy 14:3 (5*Z*, 8*Z*, 11*Z*). See Figure 1 for abbreviations.

fects signal transduction processes in human neutrophils and tumor cells in multiple ways (Nigam, S., Grierman, M., Schewe, T., Kock, J.L.F., Botha, A., Kumar, G.S., and Franke, J., submitted for publication). Thus, this novel eicosanoid may be capable of modifying inflammatory processes and mitogenesis. In view of these observations, it would be of vital interest to investigate whether 3*R*-HETE and related compounds can also be produced by those fungal strains that are pathogenic to humans.

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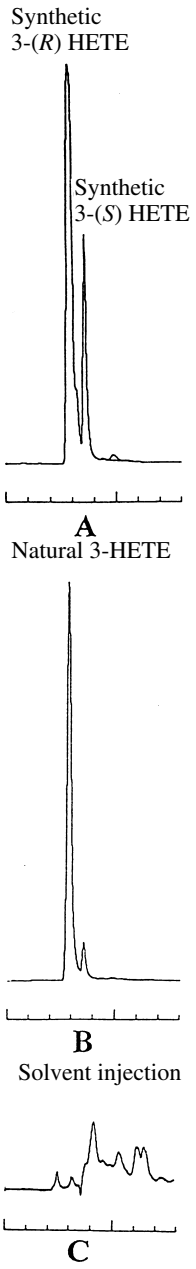

FIG. 5. Chiral phase high-performance liquid chromatography (HPLC) analysis of 3-HETE. HPLC was done with an Apex Chiral AU 50 column (4.6 × 250 mm; Jones, United Kingdom) which was developed with 1.0% isopropanol in *n*-hexane at 1.0 mL/min. The eluent was monitored by an absorbance at 208 nm. See Figure 2 for other abbreviation.

Metabolite 3-Hydroxy-5,8,11,14-eicosatetraenoic Acid (3-HETE) from the Yeast *Dipodascopsis uninucleata*, *FEBS Lett. 283,* 195–198.
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Identification of Milk Fat Triacylglycerols by Capillary Supercritical Fluid Chromatography–Atmospheric Pressure Chemical Ionization Mass Spectrometry

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ABSTRACT: Identification of milk fat triacylglycerols was accomplished by capillary supercritical fluid chromatography (SFC) combined with atmospheric pressure chemical ionization mass spectrometry [(APCI)MS]. Supercritical carbon dioxide was the carrier fluid in SFC. Ionization was achieved by introducing vapor of ammonia in methanol into the ionization chamber, which resulted in the formation of abundant $[M + 18]^+$ and $[M - RCOO]^+$ ions of triacylglycerols. These ions defined both the molecular weight and the fatty acid constituents of a triacylglycerol, respectively. SFC on a nonpolar stationary phase provided an efficient separation of triacylglycerols according to the combined number of carbon atoms in the acyl chains of a molecule. In addition to the identification of the major chromatographic peaks representing molecules with 26–54 acyl carbons, minor peaks representing triacylglycerols with an odd number of acyl carbons were separated and identified. Furthermore, compositional information on partially separated isobaric triacylglycerols, which differed substantially in the chain length of the fatty acyl residues, was achieved within some of the peaks. A new finding of the present study was the formation of abundant $[M + 18]^+$ ions of saturated triacylglycerols in addition to diagnostic fragment ions, being of primary importance in structure elucidation. This extends the applicability of capillary SFC–(APCI)MS in the analysis of both saturated and unsaturated triacylglycerols.

Lipids 32, 1285–1295 (1997).

Capillary supercritical fluid chromatography (SFC) has been shown to be a useful technique for the determination of triacylglycerols of various samples, such as berry oils (1–3), milk fat (2,4,5), and fish oil (1,2,6,7). Both nonpolar and polar stationary phases have been applied for the separation of milk fat triacylglycerols: SFC on a nonpolar stationary phase results in separation mainly according to the molecular size, i.e., the combined number of acyl carbons in the acyl chains of a triacylglycerol, whereas both the molecular size and the degree of unsaturation of a triacylglycerol affect the separation on a polar stationary phase. Capillary SFC is most often combined with a flame-ionization detector (FID), which does not allow structural elucidation of triacylglycerols.

Detailed information on molecular species compositions of triacylglycerols can only be obtained by combining chromatographic separation with detection by mass spectrometry (MS). Both high-temperature gas chromatography (GC) (8–13) and high-performance liquid chromatography (HPLC) (14–16) combined with MS have been utilized for the analysis of milk fat triacylglycerols. An ideal mass spectrum consists of ions which define both the molecular weight and the fatty acid moieties of a triacylglycerol. GC–MS with electron ionization at 70 eV results in extensive fragmentation of triacylglycerols, yielding mostly ions characterizing the fatty acid constituents of a molecule (17). The abundances of $M^{+\cdot}$ and $[M - 18]^+$ ions, which define the molecular weight, are typically low, if they are present at all. Chemical ionization (CI) is a softer ionization technique providing typically less fragmentation than electron ionization. GC–MS in ammonia positive-ion CI mode results in the formation of abundant ions containing the intact molecules, such as $[M + NH_4]^+$ ions (9), and to a lesser extent fragment ions, whereas negative-ion CI with ammonia provides only fragment ions (13). On the other hand, positive-ion CI with methane has been reported to yield only $[M - RCOOH]^+$ ions of most milk fat triacylglycerols (11). The mass spectra of milk fat triacylglycerols achieved by HPLC–MS in positive-ion CI mode, using a mixture of acetonitrile and propionitrile as a mobile phase, exhibited mostly $[MH - RCOOH]^+$ ions (15,16), whereas chloride-attachment negative-ion CI provided spectra exhibiting only $[M + Cl]^-$ ions (14). Thus, combining the data produced by positive-ion CI and negative-ion CI is in most cases needed for interpretation of the results. Other problems may be caused by the column bleed in GC–MS analyses, which may result in unreliable interpretation of spectra. In addition, high temperatures required for volatilization of triacylglycerols cause degradation or polymerization of highly unsaturated molecules (18,19). Utilization of a direct liquid inlet for

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Abbreviations: ACN, acyl carbon number; APCI, atmospheric pressure chemical ionization; (APCI)MS, atmospheric pressure chemical ionization mass spectrometry; CI, chemical ionization; FID, flame-ionization detector; GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; SFC, supercritical fluid chromatography.

interfacing HPLC to mass spectrometer requires efficient splitting of the effluent flow before introduction into the MS, which decreases sensitivity. During the last few years, interest in atmospheric pressure ionization techniques in lipid analysis has increased. Byrdwell *et al.* (20,21) were the first to report the analysis of triacylglycerols by reversed-phase HPLC combined with atmospheric pressure chemical ionization (APCI). Since then, (APCI)MS has also been applied to the identification of triacylglycerols separated by silver-ion HPLC (22).

Recently, a simple interfacing of a capillary supercritical fluid chromatograph with a mass spectrometer utilizing a commercially available HPLC–MS interface has been introduced (23). Capillary SFC–(APCI)MS of triacylglycerols, using methanol as a reactant-ion solvent, resulted in the formation of abundant $[M + H]^+$ and $[M - RCOO]^+$ ions, which characterized both the molecular weight and the fatty acid constituents of a molecule. The capillary SFC–MS method has been applied to the identification of triacylglycerols in berry oils (23,24). In our previous studies (2,5), a good separation of milk fat triacylglycerols has been achieved by capillary SFC combined with an FID on a nonpolar stationary phase. The objectives of the present research were to examine the ionization of relatively saturated triacylglycerols present in milk fat and to identify the triacylglycerols by using capillary SFC–(APCI)MS.

MATERIALS AND METHODS

Materials. Triacylglycerols of a milk sample collected after 7 d of parturition were analyzed in this study. The sample was the same (from cow 1) as described in our previous study (5). The milk fat sample was dissolved in dichloromethane (Rathburn Chemicals Ltd., Walkerburn, Scotland) to a concentration of approximately 20 mg/mL.

SFC. The SFC separations were conducted with a Lee Scientific Series 600 supercritical fluid chromatograph (Dionex, Salt Lake City, UT) combined with a Finnigan MAT TSQ-700 triple quadrupole mass spectrometer (San Jose, CA). The interfacing of the capillary SFC to the mass spectrometer was done utilizing a commercial HPLC–APCI source (Finnigan MAT) as described elsewhere (23). An electrically and pneumatically controlled Valco switching valve (Houston, TX) with an internal loop volume of 1.0 μL was used for timed split/dynamic split injections with a loop-open time of 1.0 s. Frit restrictors (30 cm × 50 μm i.d.; Dionex) were installed both at the dynamic split outlet and at the detector end of the analytical column. SFC-grade CO_2 (Scott Specialty Gases, Plumsteadville, PA) was used as a carrier fluid with a column flow rate of 0.37 mL/min measured with propane at the initial conditions of the chromatographic program with FID. An SB-Octyl-50 column (10 m × 50 μm i.d. with 0.25 μm film; Dionex) was used with a linear density programming of CO_2 from 0.140 g/mL with a rate of 0.010 g/mL/min at a constant temperature of 140°C for the elution of triacylglycerols.

MS. Triacylglycerols were ionized by using the APCI technique. Ionization of triacylglycerols was facilitated by introducing vapor of reactant-ion solvent, i.e., methanol (gradient grade; Merck, Darmstadt, Germany) or 0.5% ammonia in methanol (25% ammonia solution [p.a.-plus, Riedel-de Haën, Seelze, Germany)/methanol, 1:49, vol/vol]), into the ionization chamber *via* the sheath gas flow as reported elsewhere (23). The APCI vaporizer temperature was kept at 425°C and the APCI capillary at 200°C. The corona needle current was set at 5.0 μA. The sheath gas (nitrogen) pressure was 15 psi and the auxiliary gas (nitrogen) flow rate was 5 mL/min. The gases were preheated before being introduced into the APCI source. The mass spectrometer was operated in a full-scan mode using quadrupole 1 for scanning. Positively charged ions with *m/z* values 200–950 were scanned with a scan time of 0.8 s.

RESULTS AND DISCUSSION

Ionization of milk fat triacylglycerols. Introduction of methanol vapor into the ionization chamber in capillary SFC–(APCI)MS has been reported to yield abundant $[M + H]^+$ and $[M - RCOO]^+$ ions of unsaturated triacylglycerols of berry oils (23,24). The ions characterized the molecular weight and the fatty acid moieties of triacylglycerols, respectively. The ion abundances were affected by the molecular compositions of triacylglycerols, i.e., the degree of unsaturation, the regiospecific distribution of fatty acyl residues, and the acyl chain length and the number of double bonds and their positions in the fatty acyl residue (23,24). In general, the ion abundance of a protonated molecular ion increased with increasing degree of unsaturation of a molecule. Furthermore, triacylglycerols having 0–2 double bonds in the acyl chains were found to produce very weak or no $[M + H]^+$ ions. Instead of methanol, ammonia may be a good reactant ion for the ionization of relatively saturated triacylglycerols, as suggested in our previous study (23).

In this study, methanol vapor and 0.5% ammonia in methanol as a reactant-ion solvent were tested for the ionization of milk fat triacylglycerols analyzed by capillary SFC–(APCI)MS. The weakest possible reactant ion is formed with mixed reactant-ion solvents (25), i.e., NH_4^+ from the solution of 0.5% ammonia in methanol. The reconstructed ion chromatograms of milk fat triacylglycerols achieved by using either ammonia or methanol as a reactant-ion solvent were comparable and, thus, only the one produced by using ammonia is shown in Figure 1. Examples of the (APCI)MS spectra of milk fat triacylglycerols, produced either by methanol or by ammonia in methanol, are presented in Figure 2. The main differences are found in the molecular ion regions, whereas the $[M - RCOO]^+$ ion regions are very similar in both cases. The mass spectrum, achieved by using methanol as a reactant-ion solvent, exhibited small amounts of $[M + H]^+$ and $[M + 18]^+$ ions being only a few percentage in relative abundance (Fig. 2B). Most milk fat triacylglycerols yielded only a weak (<2%) $[M + H]^+$ ion or no ion. Reliable identification of triacylglycerols is not possible based on this information

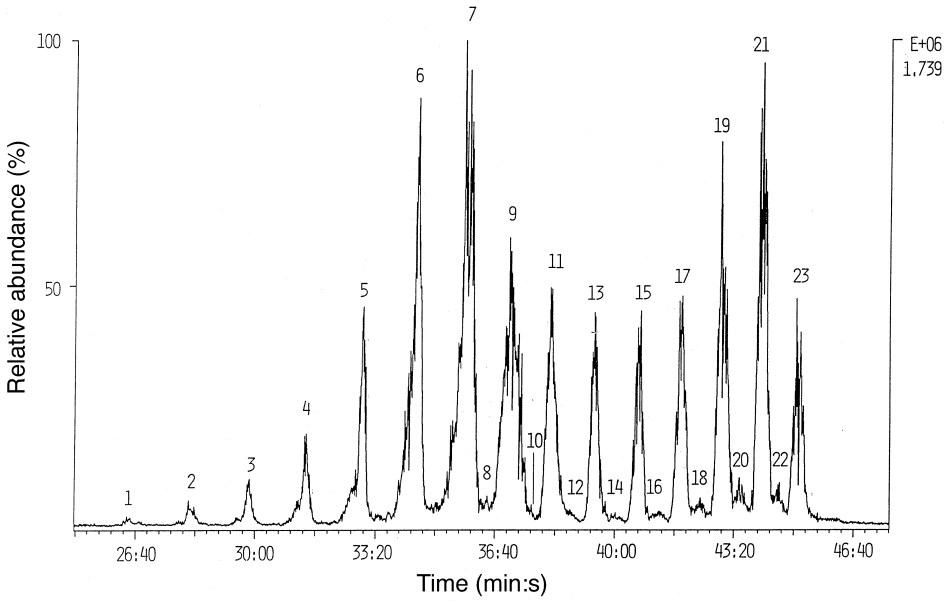

FIG. 1. Reconstructed ion chromatogram of milk fat triacylglycerols analyzed by capillary supercritical fluid chromatography–atmospheric pressure chemical ionization mass spectrometry using ammonia as a reactant ion (without smoothing).

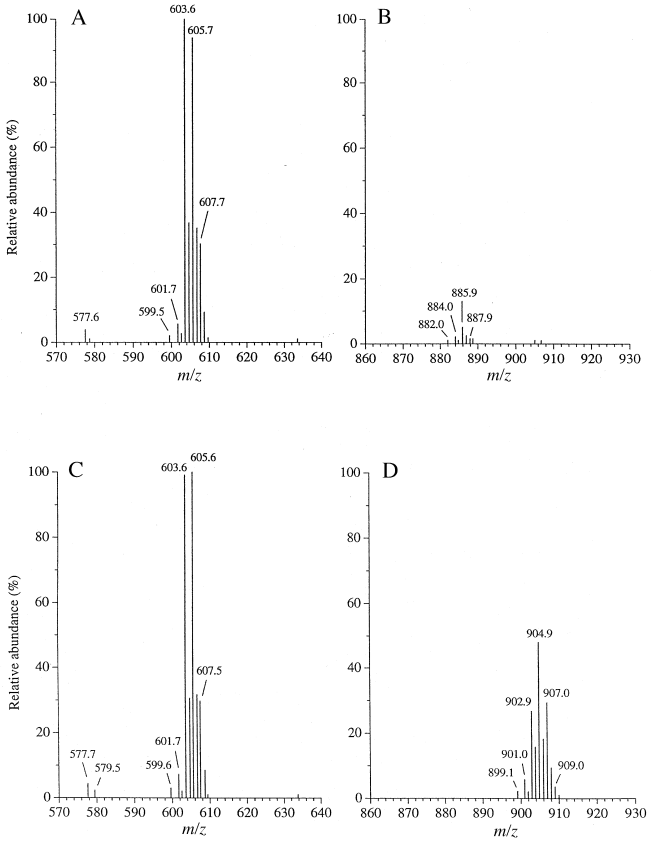

FIG. 2. Atmospheric pressure chemical ionization mass spectrometry [(APCI)MS] spectra of the chromatographic peak number 23 in Figure 1 representing milk fat triacylglycerols with 54 acyl carbons produced by using methanol (A, B) or 0.5% ammonia in methanol (C, D) as the reactant-ion solvent: (A) the region of $[M - RCOO]^+$ ions and (B) the region of $[M + H]^+$ ions produced by methanol; (C) the region of $[M - RCOO]^+$ ions and (D) the region of $[M + 18]^+$ ions produced by 0.5% ammonia in methanol.

only. The use of ammonia resulted in the formation of abundant $[M + 18]^+$ and $[M - RCOO]^+$ ions of milk fat triacylglycerols separated by capillary SFC (Fig. 2C and D). The $[M + 18]^+$ ions defined the molecular weight and, thus, the number of carbon atoms and double bonds in the acyl chains of a triacylglycerol, whereas the $[M - RCOO]^+$ fragment ions provided information on the fatty acid constituents of a molecule. The formation of $[M + 18]^+$ ions of saturated triacylglycerols is a new finding allowing reliable identification of molecular weight species of triacylglycerols.

Identification of milk fat triacylglycerols. The chromatographic resolution of milk fat triacylglycerols achieved by capillary SFC–(APCI)MS on an SB-Octyl-50 column (Fig. 1) was comparable to that reported earlier by capillary SFC-FID (2,5). The sensitivity of (APCI)MS was better than that of FID; therefore, it was possible to reduce the sample amount injected to the column. No baseline drifting was observed during the density programming of supercritical carbon dioxide.

Triacylglycerols were mainly separated according to their acyl carbon numbers (ACN; combined number of carbon atoms in the acyl chains of a triacylglycerol). Figure 2C and D show, as an example, the mass spectrum of the chromatographic peak representing triacylglycerols with 54 acyl carbons. The $[M + 18]^+$ ions defined the number of acyl carbons and the number of double bonds in a triacylglycerol: *m/z* 899.1 corresponded to the triacylglycerol 54:5, *m/z* 901.0 to 54:4, *m/z* 902.9 to 54:3, *m/z* 904.9 to 54:2, *m/z* 907.0 to 54:1, and *m/z* 909.0 to 54:0 (Fig. 2D). The presence of 54:0 in the sample was unlikely, because the ion *m/z* 909.0 abundance was contributed by the isotope ion of 54:1, containing two ^{13}C isotopes or one ^{18}O isotope. The interpretation of the $[M - RCOO]^+$ ion region was more complicated (Fig. 2C). Most of the $[M - RCOO]^+$ ions represented the loss of different fatty acyl residues from different triacylglycerols. For ex-

ample, 54:2 may yield the fragment ions *m/z* 603.6, *m/z* 605.6, and *m/z* 607.6, representing the loss of stearic acid, oleic acid, and linoleic acid, respectively. Furthermore, the loss of one fatty acid constituent from 54:3 may produce the ions *m/z* 601.7, *m/z* 603.6, *m/z* 605.6, and *m/z* 607.6, corresponding to the loss of stearic acid, oleic acid, linoleic acid, and linolenic acid. The interpretation of the mass spectra of most of the chromatographic peaks was easier compared with ACN 54 due to the presence of fewer molecular weight species in a single chromatographic peak.

Examples of the mass spectra extracted from the chromatographic peaks corresponding to triacylglycerols with ACN 26, 40, 46, and 49 are shown in Figure 3. Each mass spectrum exhibited $[M + 18]^+$ ions, which characterized the molecular weight species of triacylglycerols present in the peak. For example, peak number 1 in Figure 1 consisted of a single molecular weight species of triacylglycerol 26:0 (Fig. 3A), whereas peak number 9 represented triacylglycerols 40:0, 40:1, and 40:2 (Fig. 3B). Furthermore, the $[M - RCOO]^+$ ions provided information on the fatty acid moieties of triacylglycerols. For example, the mass spectrum of peak number 1 shows clearly the loss of fatty acids 4:0, 6:0, 8:0, 10:0, 12:0, 14:0, and 16:0 from the triacylglycerol 26:0. However, this kind of data does not provide direct information on the molecular associations of fatty acids in single triacylglycerols. The interpretation of the mass spectra of peaks number 9, 15, and 18 in Figure 1 was more complicated owing to the presence of several molecular weight species in a single chromatographic peak. Instead of information on single fatty acid moieties, the $[M - RCOO]^+$ ion region in the mass spectra consisted of ion clusters representing the loss of, for example, C_{18} or C_{16} fatty acids from the triacylglycerols. In most cases, unambiguous identification of the loss of different C_{18} fatty acids from a single triacylglycerol was not possible: for example, the ion *m/z* 495.4 in the mass spectrum of peak number 15 may be formed by the loss of 18:3 from triacylglycerol 46:3, or by the loss of 18:2 from 46:2, or by the loss of 18:1 from 46:1, or by the loss of 18:0 from 46:0 (Fig. 3C). The loss of 4:0, 6:0, and 8:0 from a triacylglycerol could be identified from each molecule. In addition to triacyl-

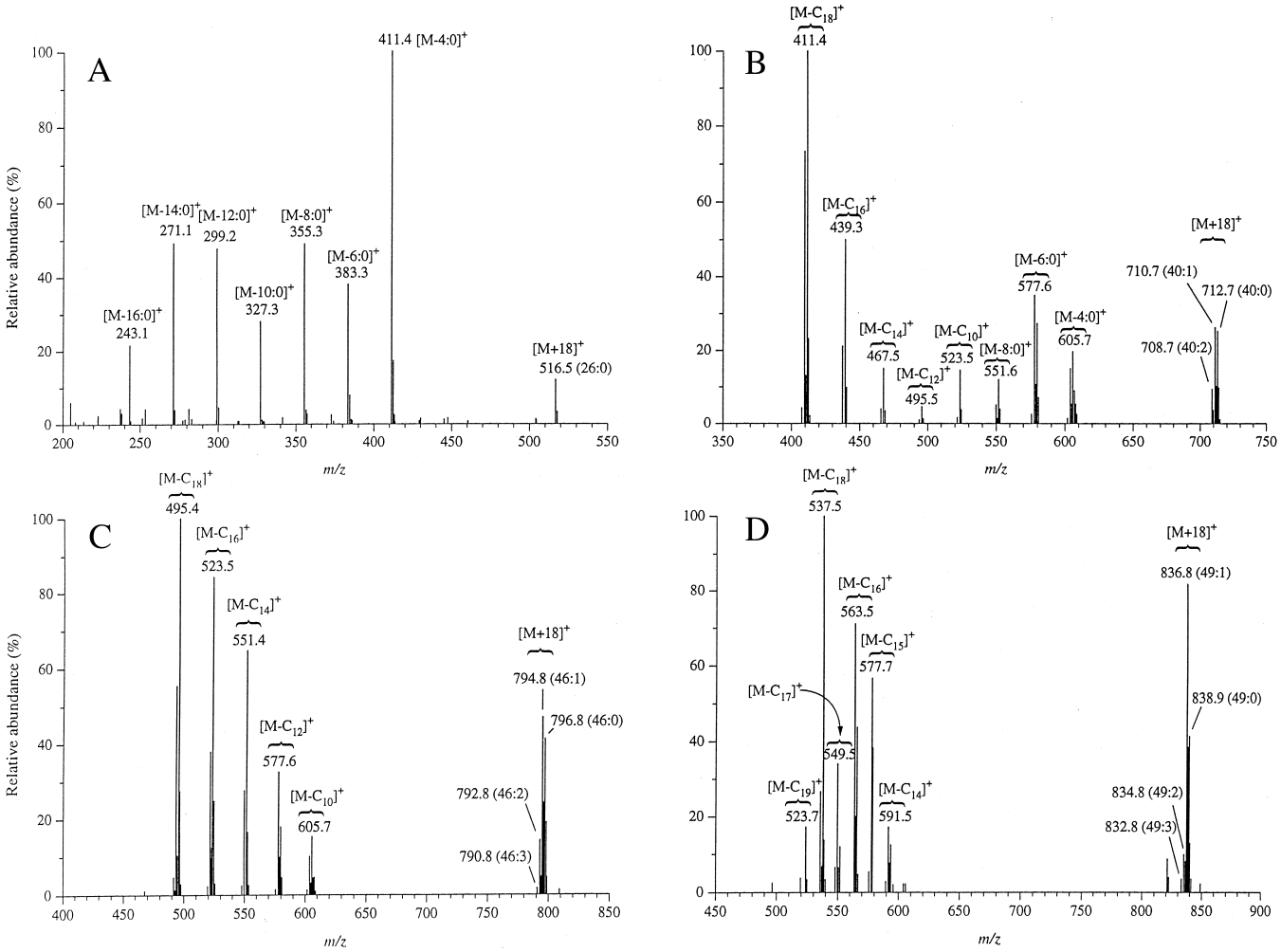

FIG. 3. Examples of the (APCI)MS spectra of four chromatographic peaks of milk fat triacylglycerols, separated by capillary supercritical fluid chromatography, representing triacylglycerols with (A) acyl carbon number (ACN) 26 (peak number 1), (B) ACN 40 (peak number 9), (C) ACN 46 (peak number 15), and (D) ACN 49 (peak number 18). Peak numbers refer to those presented in Figure 1. For other abbreviation see Figure 1.

glycerols with an even number of acyl carbons, also triacylglycerols with an odd ACN were identified. The mass spectrum of peak number 18 representing triacylglycerols with ACN 49 showed the presence of C_{15}, C_{17}, and C_{19} fatty acids in these molecules (Fig. 3D).

Previously, partial separation of isobaric triacylglycerols, differing substantially in the chain length of the fatty acyl residues, was reported on the SB-Octyl-50 column (5). Capillary SFC–(APCI)MS allowed the examination of compositional differences of the separated molecules. Figure 4A presents the mass spectrum of the whole peak number 4 in Figure 1, representing triacylglycerols 32:0 and 32:1. The possible fatty acid constituents of the triacylglycerols were 4:0, 6:0, 8:0, C_{10}, C_{12}, C_{14}, C_{16}, and C_{18} acids. The shoulder in front of the peak represented triacylglycerols which did not contain 4:0 acid (Fig. 4B). Furthermore, the relative abundances of ions corresponding to the loss of C_{12}–C_{18} acids were found compared with the total peak. According to this data, milk fat triacylglycerols with 32 acyl carbons containing 4:0 do not have other short-chain fatty acids in the same molecule. In addition, triacylglycerols containing 4:0 had stronger interaction with the column stationary phase than the others. Further evidence of the same phenomenon is provided in Figure 5, which shows the mass spectra extracted from different parts of peak number 5 in Figure 1. The fatty acid constituents of the molecules eluting in the latter part of the peak were 4:0 and C_{12}–C_{18} (Fig. 5C). In addition, there was a complete absence of C_6–C_{10} fatty acids in the spectrum, which resulted in great chain-length differences of acyl chains within a triacylglycerol and thus enhanced retention to the stationary phase. Triacylglycerols eluting in the beginning of the peak consisted of a wider range of fatty acids, i.e., from C_6–C_{18}

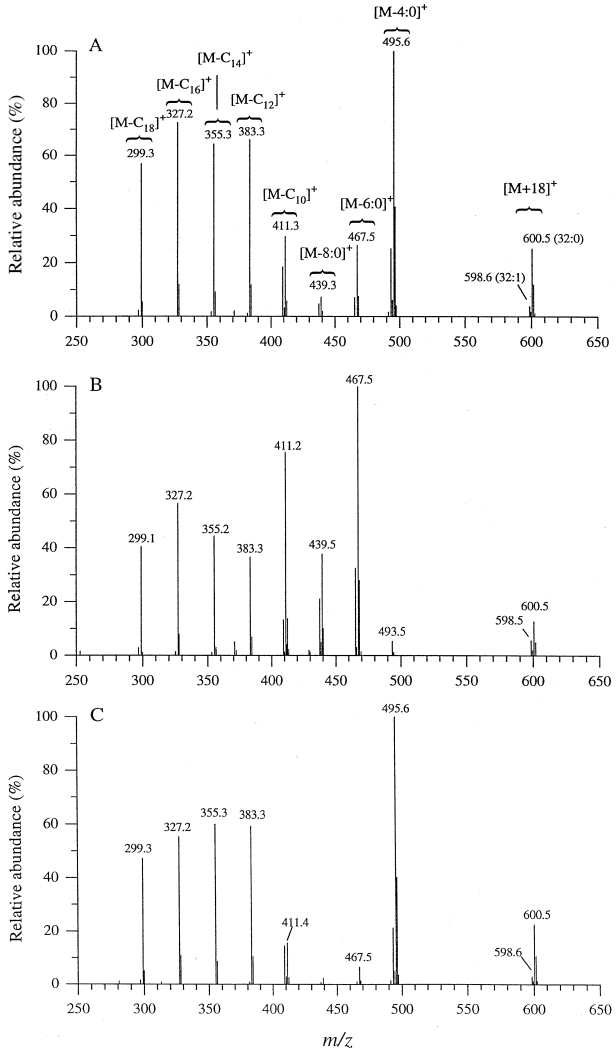

FIG. 4. (APCI)MS spectra of partially separated triacylglycerols extracted from different parts of the chromatographic peak number 4: (A) spectrum of the total peak, (B) spectrum extracted from the front shoulder of the peak, and (C) spectrum extracted from the end of the peak. Peak number refers to that presented in Figure 1. For abbreviation see Figure 1.

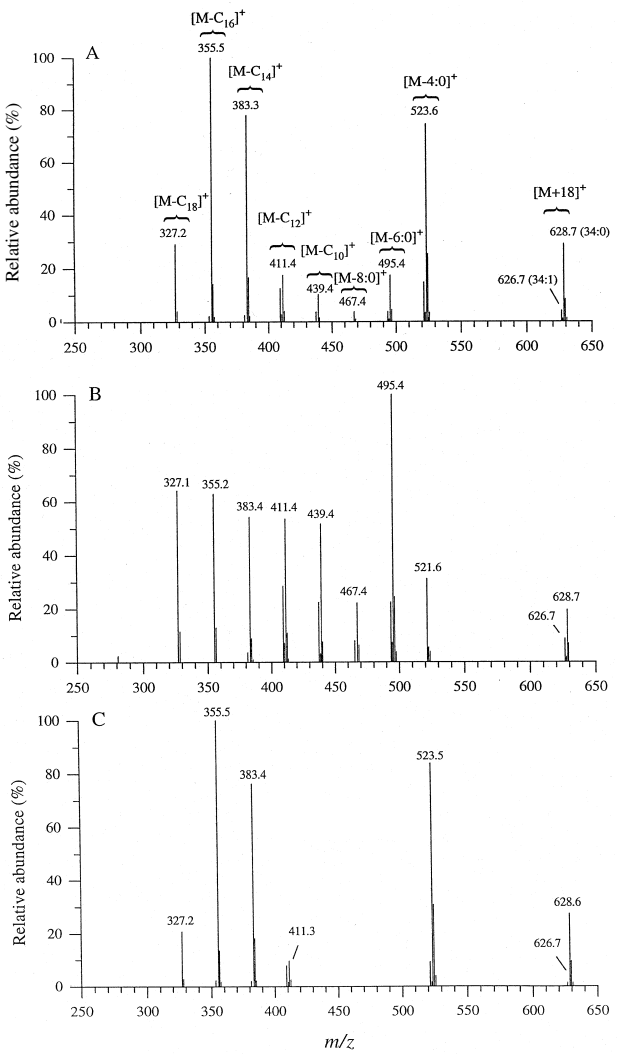

FIG. 5. (APCI)MS spectra of partially separated triacylglycerols extracted from different parts of the chromatographic peak number 5: (A) spectrum of the total peak, (B) spectrum extracted from the front shoulder of the peak, and (C) spectrum extracted from the end of the peak. Peak number refers to that presented in Figure 1. For abbreviation see Figure 1.

TABLE 1
Identification of Milk Fat Triacylglycerols Analyzed by Capillary Supercritical Fluid Chromatography–Atmospheric Pressure Chemical Ionization Mass Spectrometry

Peak number 1[a]: ACN 26[b]		Peak number 2: ACN 28			Peak number 3: ACN 30			Peak number 4: ACN 32			Peak number 5: ACN 34		
Ion	26:0[c]	Ion	28:0	28:1	Ion	30:0	30:1	Ion	32:0	32:1	Ion	34:0	34:1
m/z (abund.)	–FA[d]	*m/z* (abund.)	–FA	–FA	*m/z* (abund.)	–FA	–FA	*m/z* (abund.)	–FA	–FA	*m/z* (abund.)	–FA	–FA
243.1 (20.2)	–16:0	243.1 (14.9)	–18:0	–18:1	271.1 (34.6)	–18:0	–18:1	297.1 (2.2)		–18:0	327.2 (29.0)	–18:0	–18:1
271.1 (49.1)	–14:0	271.1 (44.8)	–16:0	–16:1	297.2 (2.7)		–16:0	299.2 (56.9)	–18:0	–18:1	353.2 (2.2)		–16:0
299.2 (47.7)	–12:0	297.0 (1.9)		–14:0	299.3 (61.3)	–16:0	–16:1	327.3 (72.5)	–16:0	–16:1	355.3 (100)	–16:0	–16:1
327.3 (28.0)	–10:0	299.2 (69.3)	–14:0	–14:1	327.1 (38.4)	–14:0	–14:1	353.2 (1.6)		–14:0	381.2 (2.5)		–14:0
355.3 (49.0)	–8:0	327.2 (15.1)	–12:0	–12:1	355.3 (34.4)	–12:0	–12:1	355.2 (64.3)	–14:0	–14:1	383.4 (77.7)	–14:0	–14:1
383.3 (38.1)	–6:0	355.2 (51.4)	–10:0	–10:1	383.2 (58.1)	–10:0	–10:1	381.4 (1.1)		–12:0	409.4 (12.5)		–12:0
411.4 (100)	–4:0	383.3 (17.9)	–8:0		409.3 (20.0)		–8:0	383.4 (63.4)	–12:0	–12:1	411.3 (17.5)	–12:0	–12:1
516.5 (12.0)	$[M + 18]^+$	409.2 (8.7)		–6:0	411.2 (25.4)	–8:0		409.3 (18.5)		–10:0	437.5 (3.8)		–10:0
		411.4 (11.9)	–6:0		437.5 (5.5)		–6:0	411.3 (29.6)	–10:0	–10:1	439.4 (10.2)	–10:0	–10:1
		437.4 (13.6)		–4:0	439.6 (29.8)	–6:0		437.4 (4.6)		–8:0	467.5 (3.8)	–8:0	
		439.4 (100)	–4:0		465.5 (24.8)		–4:0	439.5 (7.2)	–8:0		493.5 (3.9)		–6:0
		542.5 (n.d.)		$[M + 18]^+$	467.4 (100)	–4:0		465.5 (7.1)		–6:0	495.5 (17.4)	–6:0	
		544.5 (16.3)	$[M + 18]^+$		570.5 (3.1)		$[M + 18]^+$	467.4 (26.3)	–6:0		521.5 (14.7)		–4:0
					572.7 (15.8)	$[M + 18]^+$		493.4 (25.2)		–4:0	523.6 (74.2)	–4:0	
								495.5 (100)	–4:0		626.7 (4.1)		$[M + 18]^+$
								598.6 (3.8)		$[M + 18]^+$	628.8 (29.1)	$[M + 18]^+$	
								600.4 (25.2)	$[M + 18]^+$				

Peak number 6: ACN 36			Peak number 7: ACN 38				Peak number 8: ACN 39				Peak number 9: ACN 40			
Ion	36:0	36:1	Ion	38:0	38:1	38:2	Ion	39:0	39:1	39:2	Ion	40:0	40:1	40:2
m/z (abund.)	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA
353.1 (0.9)		–18:0	381.2 (3.8)		–18:0	–18:1	381.4 (10.6)		–19:0	–19:1	407.4 (4.3)			–18:0
355.3 (44.7)	–18:0	–18:1	383.2 (100)	–18:0	–18:1	–18:2	383.3 (100)	–19:0	–19:1		409.4 (73.4)		–18:0	–18:1
381.3 (4.1)		–16:0	407.3 (1.9)			–16:0	395.3 (25.7)		–18:0	–18:1	411.4 (100)	–18:0	–18:1	–18:2
383.3 (100)	–16:0	–16:1	409.3 (49.6)		–16:0	–16:1	397.5 (74.0)	–18:0	–18:1	–18:2	437.3 (21.1)		–16:0	–16:1
409.3 (16.3)		–14:0	411.3 (64.9)	–16:0	–16:1		407.2 (14.7)			–17:0	439.3 (49.9)	–16:0	–16:1	
411.4 (30.3)	–14:0	–14:1	437.4 (5.5)		–14:0	–14:1	409.3 (79.0)		–17:0	–17:1	465.5 (4.1)		–14:0	–14:1
437.4 (1.5)		–12:0	439.4 (10.9)	–14:0	–14:1		411.4 (98.9)	–17:0	–17:1		467.5 (15.0)	–14:0	–14:1	
439.4 (2.4)	–12:0	–12:1	467.5 (2.2)	–12:0	–12:1		423.3 (9.8)		–16:0	–16:1	493.5 (1.0)		–12:0	–12:1
467.4 (2.1)	–10:0	–10:1	495.5 (3.3)	–10:0	–10:1		425.5 (23.2)	–16:0	–16:1		495.5 (4.6)	–12:0	–12:1	
495.6 (2.6)	–8:0		523.6 (5.5)	–8:0			437.5 (6.1)		–15:0		521.5 (1.7)		–10:0	–10:1
521.5 (2.7)		–6:0	549.6 (9.1)		–6:0		439.5 (20.8)	–15:0			523.5 (14.5)	–10:0	–10:1	
523.7 (15.3)	–6:0		551.6 (23.1)	–6:0			521.2 (1.2)		–9:0		549.6 (5.1)		–8:0	
549.6 (20.3)		–4:0	575.7 (3.0)			–4:0	523.8 (2.0)	–9:0			551.6 (11.9)	–8:0		
551.6 (55.8)	–4:0		577.7 (43.4)		–4:0		549.3 (4.0)		–7:0		575.6 (2.6)			–6:0
654.7 (7.5)		$[M + 18]^+$	579.6 (34.3)	–4:0			551.4 (18.0)	–7:0			577.6 (34.9)		–6:0	
656.6 (25.1)	$[M + 18]^+$		680.7 (1.7)			$[M + 18]^+$	563.7 (9.2)		–6:0		579.6 (27.2)	–6:0		
			682.7 (18.7)		$[M + 18]^+$		565.6 (44.6)	–6:0			603.7 (15.0)			–4:0
			684.7 (19.9)	$[M + 18]^+$			575.8 (8.1)			–5:0	605.7 (19.7)		–4:0	
							577.5 (22.2)		–5:0		607.6 (5.4)	–4:0		
							579.5 (19.0)	–5:0			708.7 (9.3)			$[M + 18]^+$
							589.5 (17.0)			–4:0	710.7 (26.1)		$[M + 18]^+$	
							591.8 (48.7)		–4:0		712.7 (25.2)	$[M + 18]^+$		
							593.8 (20.4)	–4:0						
							694.7 (9.6)			$[M + 18]^+$				
							696.8 (20.6)		$[M + 18]^+$					
							698.7 (37.5)	$[M + 18]^+$						

(Continued)

TABLE 1 (continued)

Peak number 10: ACN 41				Peak number 11: ACN 42				Peak number 12: ACN 43				Peak number 13: ACN 44			
Ion	41:0	41:1	41:2	Ion	42:0	42:1	42:2	Ion	43:0	43:1	43:2	Ion	44:0	44:1	44:2
m/z (abund.)	–FA	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA
407.3 (16.4)			–19:0	435.2 (3.5)			–18:0	435.4 (17.3)			–19:0	465.4 (41.2)		–18:0	–18:1
409.3 (100)		–19:0	–19:1	437.3 (50.3)		–18:0	–18:1	437.3 (28.3)		–19:0	–19:1	467.6 (100)	–18:0	–18:1	–18:2
411.2 (57.4)	–19:0	–19:1		439.3 (100)	–18:0	–18:1	–18:2	439.3 (55.5)	–19:0	–19:1		491.4 (4.0)			–16:0
423.3 (34.3)		–18:0	–18:1	465.5 (19.5)		–16:0	–16:1	451.4 (24.5)		–18:0	–18:1	493.4 (47.0)		–16:0	–16:1
425.5 (96.2)	–18:0	–18:1	–18:2	467.4 (76.9)	–16:0	–16:1		453.3 (93.4)	–18:0	–18:1	–18:2	495.5 (75.0)	–16:0	–16:1	
435.5 (11.0)			–17:0	491.4 (1.4)			–14:0	463.4 (1.4)			–17:0	521.7 (14.2)		–14:0	–14:1
437.3 (52.7)		–17:0	–17:1	493.4 (16.6)		–14:0	–14:1	465.4 (28.1)		–17:0	–17:1	523.6 (39.2)	–14:0	–14:1	
439.4 (25.1)	–17:0	–17:1		495.5 (28.9)	–14:0	–14:1		467.4 (30.0)	–17:0	–17:1		549.7 (10.9)		–12:0	–12:1
451.4 (8.2)		–16:0	–16:1	521.5 (2.7)		–12:0	–12:1	477.5 (2.4)			–16:0	551.6 (27.5)	–12:0	–12:1	
453.4 (29.0)	–16:0	–16:1		523.5 (13.1)	–12:0	–12:1		479.3 (18.8)		–16:0	–16:1	575.6 (4.3)			–10:0
463.2 (1.8)			–15:0	549.6 (13.5)		–10:0	–10:1	481.4 (100)	–16:0	–16:1		577.6 (49.3)		–10:0	–10:1
465.5 (3.6)		–15:0		551.7 (39.1)	–10:0	–10:1		493.5 (26.9)		–15:0		579.6 (35.1)	–10:0	–10:1	
467.5 (22.2)	–15:0			575.5 (1.3)			–8:0	495.4 (29.5)	–15:0			603.6 (8.6)			–8:0
493.6 (3.0)		–13:0		577.6 (23.5)		–8:0		507.4 (5.0)		–14:0	–14:1	605.6 (9.6)		–8:0	
495.2 (12.4)	–13:0			579.6 (15.4)	–8:0			509.6 (16.6)	–14:0	–14:1		607.7 (2.3)	–8:0		
505.3 (1.8)			–12:0	603.6 (12.9)			–6:0	523.4 (15.0)	–13:0			764.7 (9.1)			[M + 18]$^+$
507.7 (1.4)		–12:0	–12:1	605.7 (16.0)		–6:0		535.5 (1.3)		–12:0	–12:1	766.8 (40.1)		[M + 18]$^+$	
509.4 (2.6)	–12:0	–12:1		607.6 (3.4)	–6:0			537.5 (27.4)	–12:0	–12:1		768.8 (46.1	[M + 18]$^+$		
523.6 (8.6)	–11:0			736.7 (8.2)			[M + 18]$^+$	563.9 (55.0)		–10:0	–10:1				
537.2 (8.6)	–10:0	–10:1		738.7 (30.5)		[M + 18]$^+$		565.5 (51.4)	–10:0	–10:1					
551.5 (20.7)	–9:0			740.7 (32.6)	[M + 18]$^+$			577.8 (8.7)		–9:0					
563.7 (7.7)		–8:0						579.7 (44.0)	–9:0						
565.7 (30.8)	–8:0							589.5 (4.1)			–8:0				
575.6 (3.8)			–7:0					591.6 (26.1)		–8:0					
577.5 (44.9)		–7:0						603.8 (5.2)			–7:0				
579.3 (5.5)	–7:0							605.6 (27.7)		–7:0					
591.7 (72.4)		–6:0						607.7 (20.5)	–7:0						
603.6 (15.3)			–5:0					750.7 (6.3)			[M + 18]$^+$				
605.7 (33.1)		–5:0						752.8 (57.3)		[M + 18]$^+$					
607.4 (29.8)	–5:0							754.8 (82.9)	[M + 18]$^+$						
722.4 (1.8)			[M + 18]$^+$												
724.8 (49.4)		[M + 18]$^+$													
726.8 (24.3)	[M + 18]$^+$														

(Continued)

TABLE 1 (continued

Peak number 14: ACN 45				Peak number 15: ACN 46					Peak number 16: ACN 47				Peak number 17: ACN 48				
Ion	45:0	45:1	45:2	Ion	46:0	46:1	46:2	46:3	Ion	47:0	47:1	47:2	Ion	48:0	48:1	48:2	48:3
m/z (abund.)	–FA	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA	–FA
463.3 (4.8)			–19:0	491.6 (4.7)			–18:0	–18:1	495.5 (15.4)	–19:0	–19:1		519.4 (1.3)			–18:0	–18:1
465.4 (11.5)		–19:0	–19:1	493.5 (55.4)		–18:0	–18:1	–18:2	505.5 (0.9)			–18:0	521.5 (36.2)		–18:0	–18:1	–18:2
467.3 (100)	–19:0	–19:1		495.4 (100)	–18:0	–18:1	–18:2	–18:3	507.5 (6.0)		–18:0	–18:1	523.6 (100)	–18:0	–18:1	–18:2	–18:3
479.3 (9.1)		–18:0	–18:1	519.5 (2.4)			–16:0	–16:1	509.4 (56.2)	–18:0	–18:1	–18:2	547.5 (8.4)			–16:0	–16:1
481.6 (73.9)	–18:0	–18:1	–18:2	521.6 (38.0)		–16:0	–16:1		519.5 (1.8)			–17:0	549.6 (63.0)		–16:0	–16:1	
493.6 (31.0)		–17:0	–17:1	523.5 (84.4)	–16:0	–16:1			521.2 (2.2)		–17:0	–17:1	551.7 (47.0)	–16:0	–16:1		
496.1 (93.6)	–17:0	–17:1		547.6 (2.5)			–14:0	–14:1	523.6 (100)	–17:0	–17:1		575.6 (4.4)			–14:0	–14:1
505.3 (0.9)			–16:0	549.7 (27.7)		–14:0	–14:1		533.4 (1.6)			–16:0	577.6 (62.9)		–14:0	–14:1	
507.4 (15.4)		–16:0	–16:1	551.4 (64.8)	–14:0	–14:1			535.5 (15.1)		–16:0	–16:1	579.7 (25.0)	–14:0	–14:1		
509.5 (35.6)	–16:0	–16:1		575.5 (1.4)			–12:0	–12:1	537.6 (57.7)	–16:0	–16:1		603.6 (5.9)			–12:0	–12:1
521.9 (10.8)		–15:0		577.6 (32.5)		–12:0	–12:1		547.6 (2.7)			–15:0	605.5 (7.6)		–12:0	–12:1	
523.5 (66.6)	–15:0			579.6 (17.9)	–12:0	–12:1			549.5 (55.7)		–15:0		607.7 (2.5)	–12:0	–12:1		
535.6 (8.6)		–14:0	–14:1	601.6 (1.3)				–10:0	551.5 (35.0)	–15:0			818.9 (1.7)				$[M + 18]^+$
537.7 (53.2)	–14:0	–14:1		603.6 (10.0)			–10:0	–10:1	561.5 (1.1)			–14:0	820.8 (19.9)			$[M + 18]^+$	
549.4 (19.1)		–13:0		605.7 (15.3)		–10:0	–10:1		563.5 (25.5)		–14:0	–14:1	822.8 (55.3)		$[M + 18]^+$		
551.5 (19.7)	–13:0			607.7 (4.6)	–10:0	–10:1			565.5 (37.9)	–14:0	–14:1		824.6 (39.5)	$[M + 18]^+$			
561.8 (2.1)			–12:0	790.8 (1.8)				$[M + 18]^+$	575.6 (3.6)			–13:0					
563.5 (20.1)		–12:0	–12:1	792.8 (14.3)			$[M + 18]^+$		577.6 (34.4)		–13:0						
565.5 (11.8)	–12:0	–12:1		794.8 (47.0)		$[M + 18]^+$			591.5 (6.0)		–12:0	–12:1					
577.6 (13.4)		–11:0		796.8 (41.3)	$[M + 18]^+$				593.5 (4.7)	–12:0	–12:1						
579.8 (5.9)	–11:0								806.8 (3.7)			$[M + 18]^+$					
589.6 (10.8)			–10:0						808.7 (49.0)		$[M + 18]^+$						
591.7 (19.9)		–10:0	–10:1						810.8 (85.2)	$[M + 18]^+$							
593.7 (11.9)	–10:0	–10:1															
607.6 (3.3)	–9:0																
778.9 (n.d.)			$[M + 18]^+$														
780.9 (59.0)		$[M + 18]^+$															
782.7 (44.3)	$[M + 18]^+$																

(Continued)

TABLE 1 (continued

Peak number 18: ACN 4					Peak number 19: ACN 50					Peak number 20: ACN 51					
Ion	49:0	49:1	49:2	49:3	Ion	50:0	50:1	50:2	50:3	Ion	51:0	51:1	51:2	51:3	51:4
m/z (abund.)	–FA	–FA	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA	–FA	–FA
523.7 (17.2)	–19:0	–19:1			547.5 (7.0)			–18:0	–18:1	549.6 (14.7)		–19:0	–19:1		
535.4 (26.7)		–18:0	–18:1	–18:2	549.7 (94.0)		–18:0	–18:1	–18:2	551.5 (18.8)	–19:0	–19:1			
537.5 (100)	–18:0	–18:1	–18:2	–18:3	551.8 (100)	–18:0	–18:1	–18:2	–18:3	561.5 (6.3)			–18:0	–18:1	–18:2
545.3 (1.1)				–17:0	575.6 (8.9)			–16:0	–16:1	563.6 (71.5)		–18:0	–18:1	–18:2	–18:3
547.6 (8.3)			–17:0	–17:1	577.5 (78.7)		–16:0	–16:1		565.5 (100)	–18:0	–18:1	–18:2	–18:3	
549.5 (34.0)		–17:0	–17:1		579.7 (29.7)	–16:0	–16:1			577.3 (2.5)		–17:0	–17:1		
551.7 (11.9)	–17:0	–17:1			601.6 (1.5)				–14:0	589.7 (21.5)			–16:0	–16:1	
561.5 (1.6)			–16:0	–16:1	603.6 (16.9)			–14:0	–14:1	591.7 (50.7)		–16:0	–16:1		
563.5 (71.1)		–16:0	–16:1		605.6 (19.4)		–14:0	–14:1		593.7 (9.2)	–16:0	–16:1			
565.5 (43.8)	–16:0	–16:1			607.6 (4.5)	–14:0	–14:1			603.5 (20.6)			–15:0		
575.7 (5.5)			–15:0		846.9 (3.3)				$[M + 18]^+$	605.6 (26.7)		–15:0			
577.7 (56.7)		–15:0			848.9 (33.3)			$[M + 18]^+$		619.6 (1.4)		–14:0	–14:1		
589.4 (2.9)			–14:0	–14:1	850.9 (70.2)		$[M + 18]^+$			858.9 (1.1)					$[M + 18]^+$
591.5 (17.1)		–14:0	–14:1		852.9 (24.4)	$[M + 18]^+$				861.0 (1.5)				$[M + 18]^+$	
593.6 (12.4)	–14:0	–14:1								863.0 (43.5)			$[M + 18]^+$		
605.6 (2.2)		–13:0								864.9 (67.8)		$[M + 18]^+$			
832.8 (3.5)				$[M + 18]^+$						866.9 (22.8)	$[M + 18]^+$				
834.8 (7.7)			$[M + 18]^+$												
836.8 (81.7)		$[M + 18]^+$													
838.9 (41.3)	$[M + 18]^+$														

Peak number 21: ACN 52					Peak number 22: ACN 53						Peak number 23: ACN 54						
Ion	52:0	52:1	52:2	52:3	Ion	53:0	53:1	53:2	53:3	53:4	Ion	54:0	54:1	54:2	54:3	54:4	54:5
m/z (abund.)	–FA	–FA	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA	–FA	–FA	*m/z* (abund.)	–FA	–FA	–FA	–FA	–FA	–FA
551.5 (1.0)	–20:0				573.8 (1.7)				–19:0	–19:1	577.6 (4.4)		–20:0				
575.7 (8.7)			–18:0	–18:1	575.6 (25.7)			–19:0	–19:1		579.9 (2.3)	–20:0					
577.6 (100)		–18:0	–18:1	–18:2	577.8 (16.3)		–19:0	–19:1			599.7 (3.0)					–18:0	–18:1
579.7 (48.3)	–18:0	–18:1	–18:2	–18:3	587.8 (1.1)				–18:0	–18:1	601.6 (7.3)				–18:0	–18:1	–18:2
601.7 (2.1)				–16:0	589.7 (22.4)			–18:0	–18:1	–18:2	603.7 (99.1)			–18:0	–18:1	–18:2	–18:3
603.5 (20.6)			–16:0	–16:1	591.7 (100)		–18:0	–18:1	–18:2	–18:3	605.6 (100)		–18:0	–18:1	–18:2	–18:3	
605.6 (24.0)		–16:0	–16:1		593.7 (24.0)	–18:0	–18:1	–18:2	–18:3		607.7 (29.8)	–18:0	–18:1	–18:2	–18:3		
607.9 (5.7)	–16:0	–16:1			601.5 (8.0)				–17:0	–17:1	899.0 (2.2)						$[M + 18]^+$
874.9 (4.1)				$[M + 18]^+$	605.6 (53.6)		–17:0	–17:1			900.9 (5.8)					$[M + 18]^+$	
876.9 (34.1)			$[M + 18]^+$		617.7 (8.2)			–16:0	–16:1		902.9 (26.8)				$[M + 18]^+$		
879.0 (35.0)		$[M + 18]^+$			619.5 (6.7)		–16:0	–16:1			904.9 (48.0)			$[M + 18]^+$			
881.0 (7.7)	$[M + 18]^+$				621.6 (1.0)	–16:0	–16:1				906.9 (29.4)		$[M + 18]^+$				
					886.8 (1.2)					$[M + 18]^+$	909.0 (3.6)	$[M + 18]^+$					
					889.0 (11.3)				$[M + 18]^+$								
					890.8 (58.8)			$[M + 18]^+$									
					893.0 (40.8)		$[M + 18]^+$										
					894.8 (6.5)	$[M + 18]^+$											

[a]Peak numbers refer to those presented in Figure 1.

[b]ACN = acyl carbon number.

[c]Abbreviation describes the number of acyl carbons and the number of double bonds in the acyl chains of a triacylglycerol (ACN:*n*).

[d]Loss of a fatty acid moiety (FA) from a triacylglycerol. Abbreviation: n.d., not detected.

(Fig. 5B). The ion *m/z* 521.6 was formed by the loss of 4:0 from 34:1, whereas no loss of 4:0 from 34:0 was recorded. This may be explained by a slightly weaker interaction of 34:1 with the stationary phase than 34:0. This conclusion is supported by earlier studies, where double bonds have been reported to reduce the retention of unsaturated molecules on this stationary phase (2). The same phenomenon, but to a lesser extent, is also seen in Figure 4.

Capillary SFC–(APCI)MS on an SB-Octyl-50 column resulted in separation of milk fat triacylglycerols according to their ACN (26–54). The chromatographic separation efficiency and the detection sensitivity allowed the identification of the molecular weight species and the fatty acyl residues of triacylglycerols of 23 chromatographic peaks, as presented in Table 1. The loss of possible fatty acid constituents was judged based on the fatty acid composition of milk fat triacylglycerols (5,26). Although the molecular associations of fatty acyl residues of triacylglycerols could not be determined, data on possible fatty acid constituents were achieved: for example, 4:0 acid was determined only in peaks representing triacylglycerols with ACN ≤ 40.

The major accomplishment of this study was the formation of $[M + 18]^+$ ions of saturated molecules, which is essential for the structural identification of triacylglycerols. The drawback in most studies concerning GC–MS (10–13) or HPLC–MS (15,16) of milk fat triacylglycerols has been the nearly complete absence of ions characterizing the molecular weight of intact molecules in the mass spectra. For example, Evershead (13) achieved direct information on the fatty acyl residues, based on the $RCOO^-$, $[RCOO - 18]^-$, and $[RCOO - 19]^-$ ions of milk fat triacylglycerols analyzed by GC–MS using ammonia negative-ion CI. However, the ACN identification of triacylglycerols was done according to the retention times only, owing to the absence of molecular ions in the spectra. On the other hand, some techniques, i.e., chloride-attachment negative-ion CI, provided only molecular weight information on triacylglycerols without formation of fragment ions (14). In addition to chromatography–MS combinations, milk fat triacylglycerols have been identified and quantified, for example, by fractionation of triacylglycerols by HPLC followed by analysis of the fatty acid compositions of the collected fractions by GC (27,28). A lot of valuable information has been produced by using this kind of approach, but the major drawback of these studies is the lack of information on native molecules. In the present study, a single SFC–MS determination provided information on both the molecular weights and the fatty acid constituents of saturated as well as unsaturated milk fat triacylglycerols, which is a new finding. However, the interpretation of the fatty acids was not unambiguous, owing to the elution of triacylglycerols having equivalent carbon numbers in a single chromatographic peak on an SB-Octyl-50 stationary phase. Therefore, no direct judgment of the fatty acid compositions of triacylglycerols could be done in this study. In order to simplify the interpretation of capillary SFC–(APCI)MS spectra, fractionation of milk fat triacylglycerols according to the degree of unsaturation by argentation chromatography (11,16,27) before SFC separation is recommended. Still, it is evident that several isobaric triacylglycerols remain unseparated because of the great complexity of milk fat triacyglycerols. In general, the capillary SFC–(APCI)MS method presented, utilizing ammonia in methanol for ionization purposes, is an excellent choice for the identification of milk fat triacylglycerols in comparison with the other techniques available. In this study, valuable information on the molecular compositions of milk fat triacylglycerols was achieved. In addition to the major components, structural information was also obtained from the minor components, such as triacylglycerols with odd ACN, as well as on partially separated isobaric triacylglycerols.

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Lipase-Based Quantitation of Triacylglycerols in Cellular Lipid Extracts: Requirement for Presence of Detergent and Prior Separation by Thin-Layer Chromatography

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ABSTRACT: A protocol, based on the use of *Pseudomonas* lipase, is presented to measure quantitatively the amount of triacylglycerols in extracts from cultured cells or tissues. Since the lipase also acts on di- and monoacylglycerols, separation of the extracts by thin-layer chromatography is recommended. In order to allow the lipase-catalyzed hydrolysis to proceed efficiently, lipid extracts or eluates from silica scrapings were mixed with the detergent Thesit [dodecylpoly(ethylene glycol ether)], prior to drying. After dissolution of the dried residues in water, the amount of triacylglycerols was quantified using *Pseudomonas* sp. lipase, glycerol kinase, glycerol-phosphate oxidase, and peroxidase. The activity of the latter enzyme was followed either colorimetrically in the presence of 4-aminoantipyrine and 2,4,6-tribromo-3-hydroxybenzoic acid or fluorimetrically in the presence of homovanillic acid.
Lipids 32, 1297–1300 (1997).

During the course of our investigations on the role of androgens in prostate cancer, accumulation of intracellular lipid droplets was observed in androgen-stimulated LNCaP cells, cells derived from a lymph node carcinoma of human prostate. Based on thin-layer chromatography (TLC) analysis, combined with enzymatic measurements, these lipids appear to be cholesteryl esters and triacylglycerols (TAG) (1). Routine procedures for the quantitation of TAG are nowadays based on enzymatic methods. By means of lipase (sometimes combined with esterase), TAG are hydrolyzed and the amount of liberated glycerol is monitored by indicator reactions. A convenient indicator reaction, incorporated in some commercial kits, is based on the combined use of glycerol-kinase and glycerol-3-phosphate oxidase whereby the produced H_2O_2 is captured by peroxidase in the presence of suitable H donors (2–4). When trying to measure the amount of TAG in lipid extracts from LNCaP cells, directly or after TLC separation, by means of a commercial kit [Sigma triglyceride (GPO-Trinder) reagents; Sigma Chemical Co., St. Louis, MO], some problems were encountered. Such kits are intended for the analysis of serum in which TAG are bound to lipoproteins. Despite the presence of surface-active compounds in the assay medium, dissolution of dried TAG was not adequate as revealed by the use of triolein standards. Based on further investigations on the use of detergents to solubilize the dried lipids and on the specificity of the lipase, an improved colorimetric or fluorescent procedure was developed to measure TAG in cellular lipid extracts.

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Abbreviations: AAP: 4-aminoantipyrine; LNCaP, lymph node carcinoma of the prostate; TAG, triacylglycerol; TBHB: 2,4,6-tribromo-3-hydroxybenzoic acid; TLC, thin-layer chromatography.

EXPERIMENTAL PROCEDURES

Cell culture and lipid extraction. LNCaP cells were cultured as described in Ref. 1. After washing and scraping the monolayers in phosphate buffered saline, cells were collected by centrifugation, resuspended in 0.8 mL of phosphate buffered saline, and extracted with chloroform/methanol using 1 M NaCl during phase separation (5). Aliquots of the organic extract were analyzed for phospholipids (5) and for TAG, either directly or after separation by TLC on silica G 60 plates (0.25 mm thickness; Merck, Darmstadt, Germany) using heptane/diethyl ether/acetic acid (60:40:1, by vol) as solvent. Lipids were transiently visualized with iodine, and spots corresponding to TAG were scraped into glass tubes and eluted quantitatively with 2 mL of chloroform/methanol (1:1, vol/vol).

Colorimetric TAG assay. Trioleoylglycerol standards and aliquots of the cellular lipid extracts or of the silica eluates, freed from silica particles by centrifugation, containing up to 40 nmoles of TAG, were mixed with 10 μL of 20% (wt/vol) of the detergent Thesit [dodecylpoly(ethylene glycol ether)] (Boehringer, Mannheim, Germany) (in chloroform) and dried. To the oily residue was added 50 μL of water, tubes were placed in a shaking water bath for 10 min to allow dissolution of the lipids, and reactions were started by adding 450 μL of assay mixture and incubated at 30°C. Final concentrations were 0.1 M Tris pH 7.5, 2 μM FAD, 1 mM ATP, 3 mM $MgCl_2$, 2 mM 4-aminoantipyrine (AAP), 8 mM 2,4,6-tribromo-3-hydroxybenzoic acid (TBHB) (Research Organics, Cleveland, OH), 8 U/mL *Pseudomonas* sp. lipase (Sigma), 1 U/mL glycerol kinase (*Candida mycoderma*; Boehringer), 2 U/mL glycerol-3-phosphate oxidase (Boehringer), and 8 U/mL peroxi-

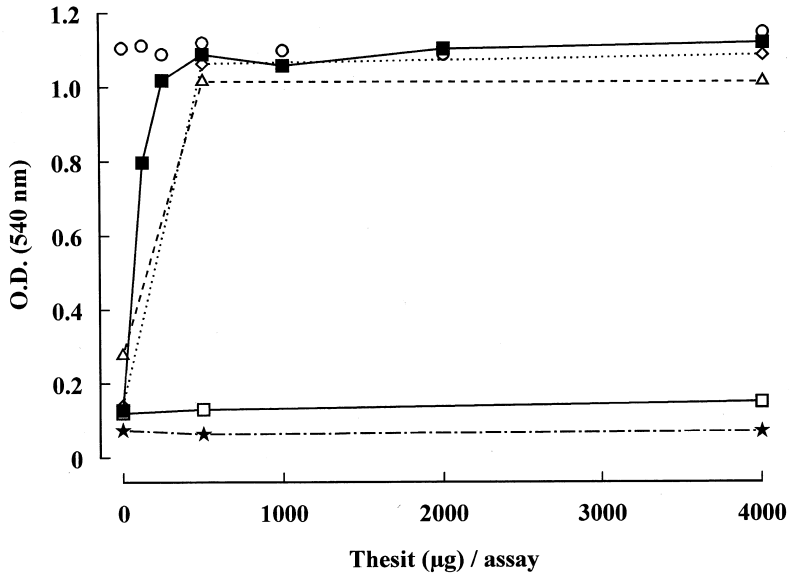

FIG. 1. Influence of the detergent dodecylpoly(ethylene glycol ether) (Thesit; Boehringer, Mannheim, Germany) on the quantitation of triacylglycerols. Chloroform solutions, containing 50 nmoles of trioleoylglycerol (■—■), 1,2-dipalmitoylglycerol (◇—◇), and 1-monooleoylglycerol (△—△) were pipetted at the bottom of glass tubes and mixed with the indicated amount of Thesit, dissolved in chloroform, before drying. After dissolution of the lipids in 50 µL of water, reactions were started by adding 0.45 mL of assay mixture [reconstituted Sigma GPO-Trinder reagents (Sigma Chemical Co., St. Louis, MO)]. After incubation at 30°C for 30 min, absorbance was measured at 540 nm. When the glycerides solutions were dried in the absence of detergent, followed by solubilization in 50 µL of water containing the indicated amounts of Thesit, almost no hydrolysis occurred (only shown for 50 nmoles of trioleoylglycerol; (□—□). No influence of the detergent was seen on the color formation either when using 50 nmoles of L-glycerol-3-phosphate (○) or of glycerol standards (data not shown), both being quantitatively converted, or on blanks (★—★).

dase (horseradish; Boehringer). Full-color development was achieved after 45 min. When relying on the reported ε value (Ref. 6; 28,000 $M^{-1} \cdot cm^{-1}$ at 511 nm), we recommend to read absorbances after 1 h of incubation.

Fluorimetric TAG assay. Trioleoylglycerol standards and aliquots of the cellular lipid extracts or silica eluates, containing up to 10 nmoles of TAG, were mixed with 5 µL of 20% (wt/vol) Thesit (dissolved in chloroform), dried, and dissolved in 50 µL of water. After dissolution, 150 µL of reaction mixture was added. Final concentrations were exactly as described for the colorimetric assay except that AAP and TBHB were replaced by 1.2 mM homovanillic acid. After incubation at 30°C for 1 h, reactions were quenched by adding 2 mL of 0.5 M carbonate buffer pH 10.7 containing 10 mM EDTA (7) and fluorescences were read in an Aminco SPF500 Fluorimeter (excitation at 327 nm; slit 2 nm; emission at 420 nm; slit 8 nm) (American Instruments Company, Silver Spring, MD). Standardization of the readings by means of uric acid/urate oxidase (7) indicated full conversion of the triolein to homovanillic acid dimer.

RESULTS AND DISCUSSION

To study the nature of the neutral lipid accumulation in androgen-treated LNCaP cells (1), a commercial kit [Sigma triglyceride (GPO-Trinder) reagents] was evaluated for the analysis of TAG in lipid extracts prepared from these cells. No color formation was observed, however. Since we also saw no response with dried trioleoylglycerol standards, we investigated the use of detergents to solubilize the dried lipids before assaying. Addition of cholate, reported to stimulate lipase (4), and some nonionic detergents [Triton X-100, Thesit, octylglucoside, and octanoyl-*N*-methylglucamide (all from Boehringer)] was not effective, although glycerol or glycerol-3-phosphate standards were quantitatively measured in the presence of these detergents. The addition of Thesit, dissolved in chloroform, to the standards and lipid extracts prior to drying, resulted in effective dissolution in an aqueous medium and hydrolysis of the TAG. As shown in Figure 1, the extent of hydrolysis was dependent on the amount of Thesit added. Although it has been claimed that *Pseudomonas* sp. lipase (the enzyme present in the Sigma kit) only acts on TAG (in the presence of cholate and isotridecanol polyglycol ether) (8), our data show that diacylglycerols (1,2-dipalmitoyl-*sn*-glycerol and 1,3-dioleoyl-*sn*-glycerol) and monoacylglycerols (1-monooleoyl-*rac*-glycerol, 3-monopalmitoyl-*sn*-glycerol) also are quantitatively converted to glycerol (Fig. 1; Fig. 2; Van Veldhoven, P.P., unpublished data). Hence, when applied to crude tissue or cell extracts or sera, TAG levels might be overestimated (see below).

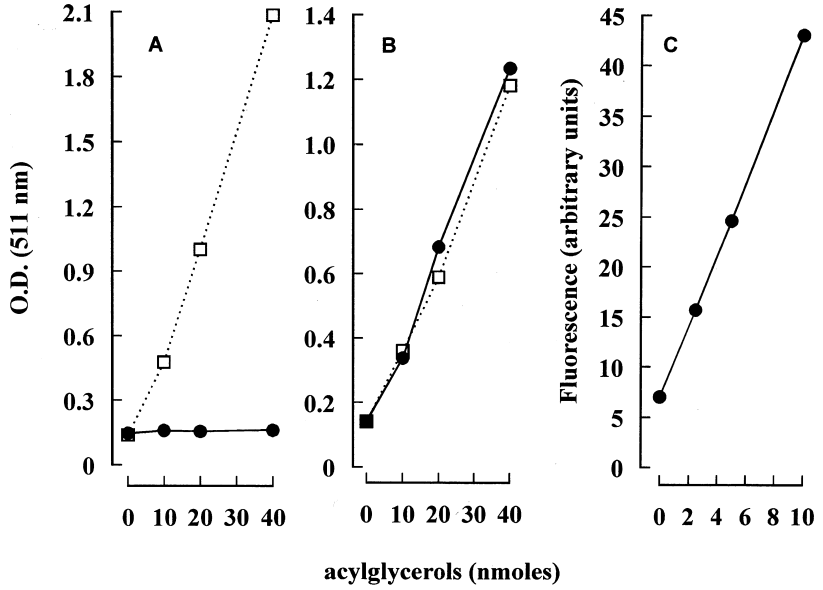

FIG. 2. Comparison of lipase activity on trioleoylglycerol in the presence of Thesit and linearity in function of trioleoylglycerol concentration in colorimetric and fluorimetric assays. (A) Indicated amounts of trioleoylglycerol (●) and 1-monopalmitoylglycerol (□) were mixed with 10 µL of 20% (wt/vol) Thesit (in chloroform) and processed as described in the Experimental Procedures section [colorimetric triacylglycerol (TAG) assay], but using *Rhizopus arrhizus* lipase (Boehringer) (final concentration 50 U/mL) and O.D. were measured after 20-min incubation. Full-color formation requires longer incubation times, being 25 min. (B) As panel A, except that the reaction mixture contained *Pseudomonas* sp. lipase (Sigma) (final concentration 8 U/mL). O.D. were measured after 20 min of incubation. (C) Indicated amounts of trioleoylglycerol (●) were analyzed as described in the Experimental Procedures section (fluorimetric TAG assay). See Figure 1 for other abbreviations and company sources.

In order to be able to analyze the influence of different androgen analogs using fewer cultured cells, we subsequently tried to increase the sensitivity of the assay. For the Trinder reaction, AAP and *N*-ethyl-*N*-(3-sulfopropyl)-*m*-anisidine are used in the kit, resulting in an ε value of 11,000 $M^{-1} \cdot cm^{-1}$ (for glycerol). Substituting the phenol (or its derivative) by TBHB results in a better sensitivity (9). In an indicator reaction, formulated on the basis of commercially obtained enzymes and chemicals, using optimized concentrations of TBHB and AAP (6), an extinction coefficient of 28,000 $M^{-1} \cdot cm^{-1}$ was obtained (for glycerol and L-glycerol-3-phosphate). Two lipase preparations were tested, one from *Rhizopus arrhizus*, the other one from *Pseudomonas* sp. Both lipases hydrolyzed monoacylglycerols, but as shown in Figure 2 (panels A and B), only the (more expensive) *Pseudomonas* enzyme was active on TAG in the presence of Thesit. If still higher sensitivities are required, the indicator reaction is easily adapted to a fluorimetric determination of the formed hydrogen peroxide by replacing AAP/TBHB by homovanillic acid (10) (Fig. 2C). In a representative experiment the levels of TAG (expressed per 100 nmoles of phospholipids to correct for possible losses during extraction) as determined directly in lipid extracts of LNCaP cells with the fluorimetric procedure were 11.9 ± 0.25 nmol (mean ± SEM of determinations on three separate culture dishes). After treating the cultures with 10^{-8} M R1881 (methyltrienolone; Dupont-New England Nuclear, Brussels, Belgium) during 4 d, the levels increased to 69.2 ± 5.0 (n = 3) nmol. If the extracts were first subjected to TLC, these values decreased by about 30%, being 9.21 ± 0.21 and 49.5 ± 1.9 nmol per 100 nmoles of phospholipids. This decrease is consistent with the levels of di- and monoacylglycerols present in these cells and that were measured, after TLC separation, in an analogous manner. In later experiments, we found that the basal levels of TAG in LNCaP cells can vary considerably. Contributing factors appear to be the culture conditions (different batches of serum) and cell passage number (Esquenet, M., Swinnen, J.V., Verhoeven, G., and Van Veldhoven, P.P., unpublished data).

Summarizing, in order to quantify enzymically the mass of TAG in extracts from tissues or cultured cells, we recommend an initial separation of the lipid extracts by TLC. To allow an efficient hydrolysis by *Pseudomonas* lipase, the silica eluates are dried in the presence of Thesit. The formed glycerol is measured by means of glycerol-kinase, glycerol-3-phosphate oxidase and peroxidase, either colorimetrically using AAP/TBHB or fluorimetrically. Owing to the nonspecificity of the lipase, similar approaches can be used to measure other glycerol esters such as mono- and diacylglycerols.

ACKNOWLEDGMENT

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Biochemistry, Chemistry, and Function of Steroids

Edward J. Parish and W. David Nes

This issue of *Lipids* contains a collection of research and review articles which resulted from papers presented at the 88th American Oil Chemists' Society's (AOCS's) Annual Meeting & Expo in Seattle, Washington, at the Washington State Convention & Trade Center on May 11–14, 1997. A number of internationally known speakers participated in the symposium on "Biochemistry, Chemistry, and Function of Steroids" which was held on Monday, May 12. This symposium was sponsored by the Biotechnology Division of the AOCS. The program included topics on nitrogen-containing steroids, sterol methylation reactions with an emphasis on drug design and testing, sterols of primitive marine fungi, the role of *N*-nitroso bile acid amides in gastrointestinal cancer, synthesis and molecular modeling in the area of brassinosteroid research, recent developments in the field of plant steroid hormones, and the remote functionalization of steroid side chains with chromyl acetates. The symposium was very successful, and the topics presented were the focus of lively discussions at the end of each presentation. These topics have been expanded upon and are presented in this issue of *Lipids*. It is our hope that these papers will stimulate further research activities in the steroid field.

Recent Developments in the Field of Plant Steroid Hormones

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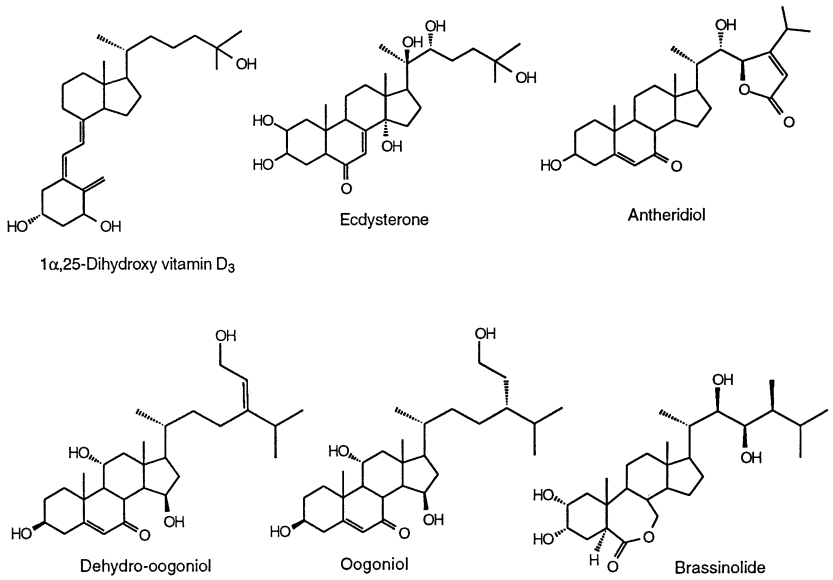
ABSTRACT: Brassinolide and related brassinosteroids are a novel group of steroids which appear to be ubiquitous in plants. There is compelling evidence, particularly from recent genetic studies, that these steroids are essential for normal plant growth and development. Synthesis of brassinosteroids and aspects of their biochemistry are reviewed.
Lipids 32, 1303–1308 (1997).

Because of their great importance in the function and structure of animals and plants, steroids, more than any other group of low molecular weight natural products, have commanded the interest of organic chemists for many decades. Much of the effort in synthesis of steroids occurred from about 1940, following the discovery of mammalian adrenocortical and sex hormones (1). In more recent times, there has been renewed interest in synthesis because of the isolation of new steroids possessing functionalized side chains. These include 25-hydroxy vitamin D_3 (calcidiol), 1α, 25-dihydroxy vitamin D_3 (calcitriol), and analogs of these hormones (2); ecdysterone and other insect molting hormones (3), a large group which are also found in plants (3); fungal sex hormones antheridiol, deoxyantheridiol, dehydro-oogoniol and oogoniol (4); and the relatively new class of steroids, the plant growth hormone brassinolide, and related brassinosteroids (5) (Scheme 1).

A remarkable feature of these hormones is that they retain the side chains of their biosynthetic precursors, i.e., cholesterol, fucosterol, and campesterol. This is in contrast to adrenocortical and sex hormones where the side chain is completely or almost completely missing. It turns out that the stereochemistry of the side chain is important for biological activity. Therefore, the challenge for synthetic chemists is to construct these molecules stereochemically pure.



SCHEME 1

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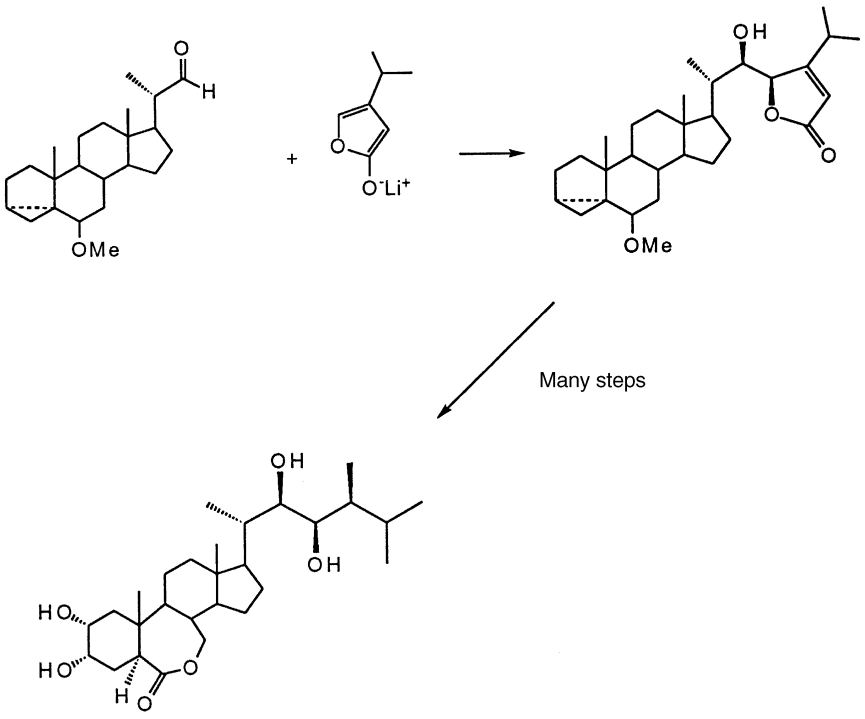
Abbreviations: CPD, constitutive photomorphogenesis and dwarfism; DET2, de etiolated.



SCHEME 2

We and other investigators have found that the most practical way to build side chains of fungal and plant hormones is to start from readily available compounds such as 17-keto steroids, 20-keto steroids, or steroids with a C_{22} aldehyde function. These are generally prepared from abundant plant or animal sterols, e.g., stigmasterol, ergosterol, sitosterol, and cholic acids. We have succeeded in synthesizing antheridiol, dehydro-oogoniol, oogoniol (6), and brassinolide (7) in this way. Because of widespread interest in brassinolide and related steroids, we have continued our synthetic studies and have collaborated with other investigators in biological studies of brassinosteroids.

Elucidation of the structure of brassinolide was first reported in 1979 (8). This achievement was the result of many years' effort by John W. Mitchell and other scientists at the USDA laboratories in Beltsville, Philadelphia, and Peoria to identify the substance in pollen of the rape plant *Brassica napus* responsible for accelerated growth of internode sections of bean plants. The so-called bean second-internode assay had been developed for detecting gibberellins. Brassinolide was extremely active, and at a concentration of 0.1 μg per plant it caused the unique effect of splitting the internode.

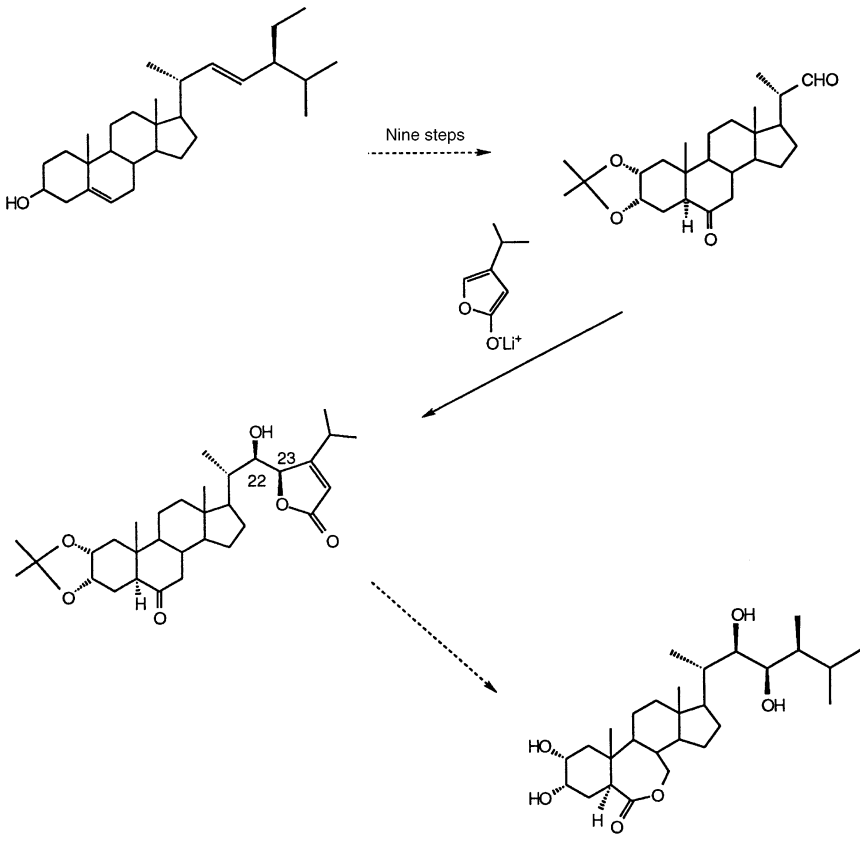
In the past 18 yr, over 60 analogs of brassinolide (brassinosteroids) have been detected in a wide variety of plants. Brassinolide remains the most biologically active brassinosteroid. It acts at very low concentrations (10^{-9} M) in eliciting cell elongation and/or proliferation, and showing strong synergistic interactions with auxin and additive interactions with gibberellins, in many test systems (5).

Synthetic studies. In our first synthesis of brassinolide, the key step was condensation of a C_{22} aldehyde with the anion derived from 3-isopropylbut-2-enolide (Scheme 2). The product was then converted into brassinolide by manipulation of functional groups on the side chain and on the tetracyclic nucleus (7) (Scheme 2).

We improved our synthesis by employing an aldehyde containing 2α,3α-dihydroxy and 6-keto functional groups (9). It was readily prepared in nine steps from stigmasterol as illustrated in Scheme 3.

A noteworthy feature of the aldol condensation of the aldehyde with the anion from isopropylbutenolide is that the main product was obtained with desired 22*R*, 23*R* stereochemistry and in high yield. The side chain could then be converted into the brassinolide side chain in five steps. The overall yield of brassinolide from stigmasterol was 7% (9). Many grams of pure brassinolide have been obtained using this method. Several syntheses of brassinolide have also been reported by other groups (10,11).

Among the naturally occurring brassinosteroids, 24-epibrassinolide and 28-homobrassinolide have attracted the interest of synthetic chemists. They possess structures which should, in principle, be readily prepared from the abundant ergosterol and stigmasterol, respectively. However, hydroxylation of the side chains of these steroids with osmium tetraoxide gives mainly the undesired 22*S*, 23*S* product. By

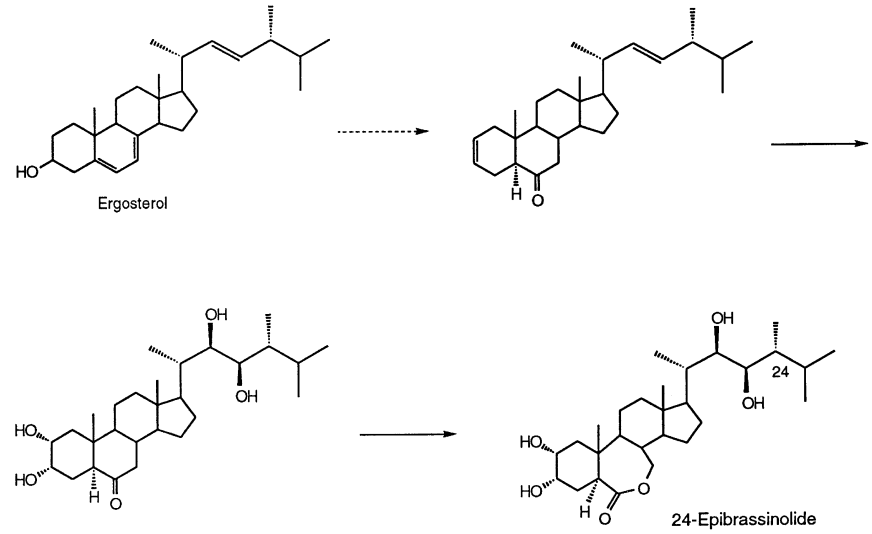


SCHEME 3

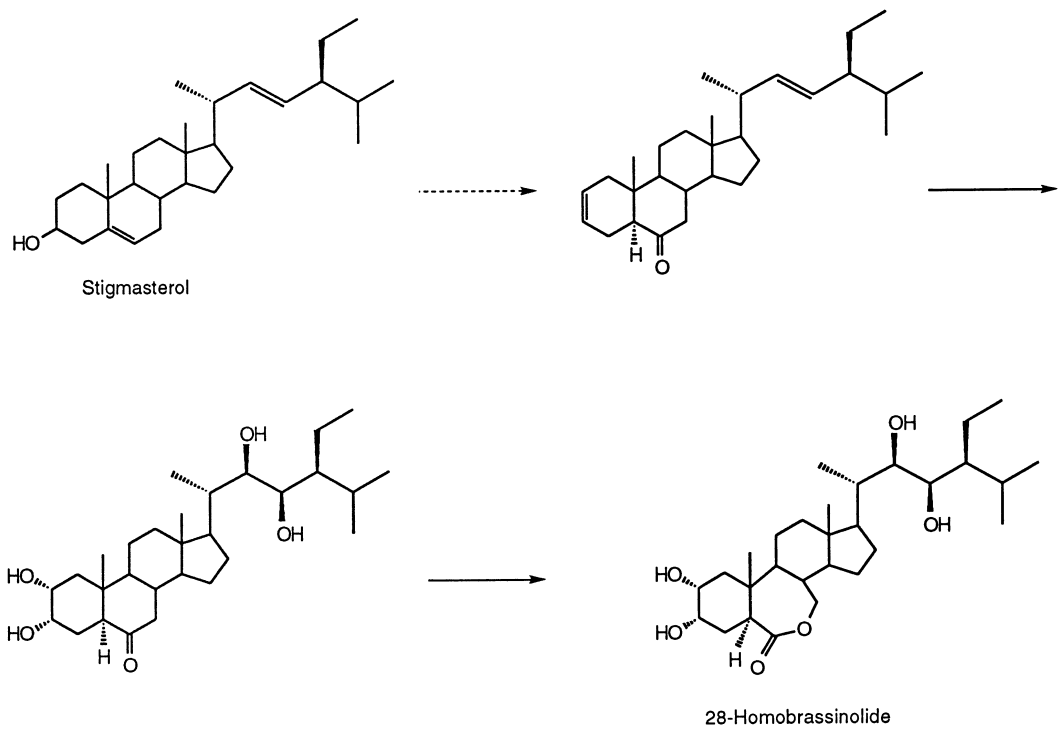
employing the method of asymmetric dihydroxylation, first reported by Sharpless *et al*. (12) [O_sO_4, $K_3Fe(CN)_6$ with the chiral ligand dihydroquinidine 4-chlorobenzoate in 2-methyl-2-propanol] the desired 22*R*,23*R* products were obtained in high yield (Schemes 4 and 5) (13,14).

Several groups besides ours have accomplished practical syntheses of naturally occurring brassinosteroids which are now commercially available (10,15–17).

Structure–activity relationships. Having prepared several brassinosteroids and their isomers, it was of interest to exam-



SCHEME 4



SCHEME 5

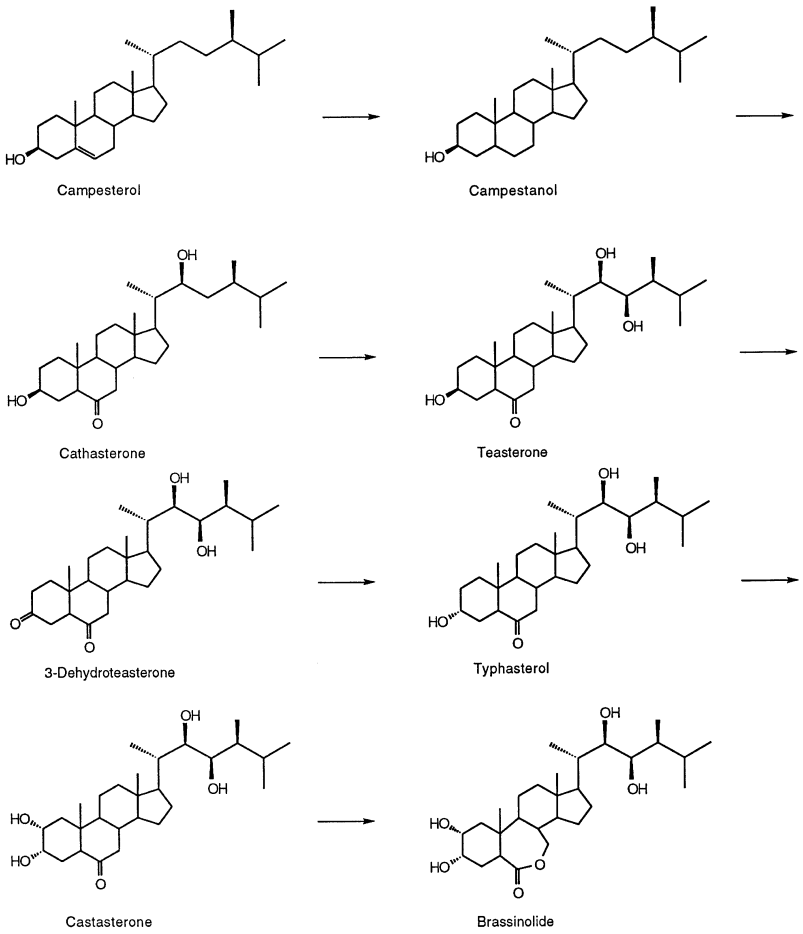
ine and compare their biological activity. These studies were carried out by my colleague, Professor Steven Clouse, using the soybean epicotyl elongation assay. Brassinolide and 28-homobrassinolide were found to elicit similar activity over a range of concentrations (Fig. 1) although brassinolide was more effective at lower concentrations. Homobrassinolide was also compared with 24-epibrassinolide, 22*S*, 23*S*-24 epibrassinolide and 22*S*, 23*S*-homobrassinolide. The first two steroids had similar activity to homobrassinolide, but 23*S*, 23*S*-homobrassinolide was substantially less active at all concentrations tested (14).

In seeking an explanation for this lower activity, we examined the molecular structures of the five steroids by comparing energy-minimized conformations for differences in nonbonded distances between various atoms in the side chains. A reference point, C_{16}, was selected on the rigid tetracyclic skeleton and distances between C_{16} and various atoms were determined. The results show that, apart from the C_{16}/C_{22} and C_{16}/C_{28} distances, all computed distances for 22*S*, 23*S*-homobrassinolide differ substantially more from the corresponding distances for brassinolide than is the case with the other isomers. These differences may be reflected in the poorer fit of 22*S*, 23*S*-homobrassinolide into the active site of a receptor for the hormone, thus resulting in lower biological activity.

Molecular genetic studies. Both plant and animal hormones effect gene expression before onset of cellular and physiological changes. Recently Zurek and Clouse (18) have shown that brassinosteroids regulate expression of specific genes in soybean hypocotyls and epicotyls and whole plants of *Arabidopsis*. The first cloning of a gene regulated specifically by brassinolide has been accomplished. This brassinosteroid upregulated gene has extensive homology with genes in other plants that code for xyloglucan endotransglycosylases which mediate cell wall loosening.

Other evidence of gene expression mediated by brassinolide comes from examination of biosynthetic intermediates between campesterol and brassinolide (Scheme 6). This scheme has been defined by several groups in Japan (19). A mutant of *Arabidopsis* has been described (DET2: de etiolated) which, when grown in light, results in an extremely dwarfed phenotype, and other changes. The gene (DET2) involved in this loss-of-function mutation has been cloned by Li *et al.* (20) and found to possess a surprising homology to mammalian steroid 5α-reductases that catalyze the NADPH-dependent conversion of testosterone to dihydrotestosterone. It has been proposed that the DET2 gene is required for brassinolide biosynthesis, specifically for the reduction of campesterol to campestanol. Feeding experiments with the DET2 mutant have shown that brassinolide rescued the DET2 phenotype to wild type in a dose-dependent manner both in the light and the dark.

Another mutant of *Arabidopsis* has been studied with a phenotype similar to DET2 (but nonallelic). This mutant (CPD: constitutive photomorphogenesis and dwarfism) has been found to have a gene (CPD) which encodes a protein with 50–60% sequence identity with conserved domains of microsomal cytochrome P 450 monoxygenases (21). The CPD gene appears to encode another enzyme in brassinolide



SCHEME 6

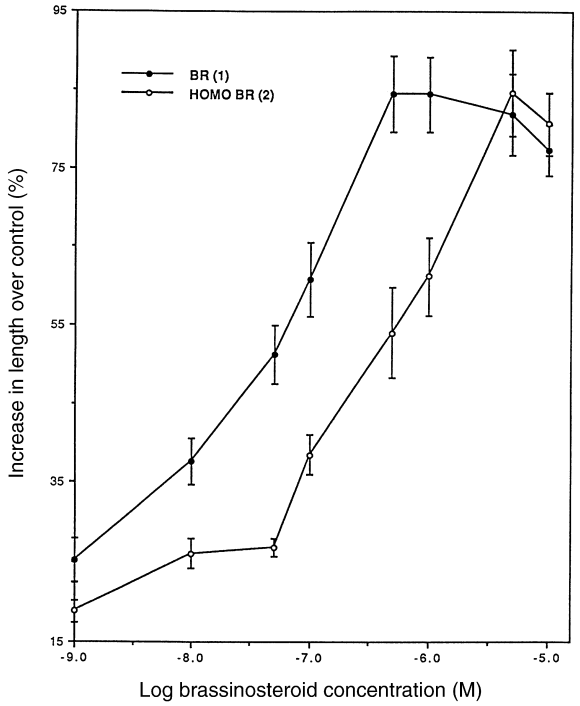


FIG. 1. Effect of brassinolide (BR) and homobrassinolide (HOMO BR) on soybean epicotyl elongation. Twenty replicate epicotyl sections (1.5 cm) were auxin-depleted in KPSC buffer (10 mM potassium phosphate, pH 6.0, 2% sucrose, 50 µg/mL chloramphenicol) for 2 h followed by incubation in the indicated compound for 19 h. Error bars are ± SE (14).

biosynthesis, this time involving a hydroxylation step. When the cpd mutant was grown in the presence of C_{23} hydroxylated brassinolide precursors including teasterone, 3-dehydroteasterone, typhasterol and castasterone, it was completely rescued to wild-type phenotype in light or darkness. However, cathasterone and its precursors which lack a hydroxyl at C_{23}, failed to rescue the CPD mutant, suggesting that the CPD protein causes hydroxylation of cathasterone to teasterone. These experiments clearly demonstrate the importance of brassinolide in the growth and differentiation of *Arabidopsis*. Brassinosteroids are now established as a new class of plant hormones.

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Sterols of the Phylum Zygomycota: Phylogenetic Implications

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ABSTRACT: The sterol composition of 42 fungal species representing six of the eight orders of the Zygomycota was determined using gas–liquid chromatography–mass spectrometry to assess whether the distribution of major sterols in this phylum has taxonomic or phylogenetic relevance. Ergosterol, 22-dihydroergosterol, 24-methyl cholesterol, cholesterol, and desmosterol were detected as the major sterols among the species studied. Ergosterol was the major sterol of the Dimargaritales, Zoopagales, and 13 of the 14 Mucorales families included in this study. Desmosterol appeared to be the characteristic sterol of the Mortierellaceae (Mucorales). 24-Methyl cholesterol was the major sterol of the Entomophthorales genera *Entomophthora*, *Conidiobolus* and *Basidiobolus*, but cholesterol was the sole sterol detected in *Delacroixia coronatus*. The Kickxellales species analyzed in this study were characterized by 22-dihydroergosterol as the major sterol. These results suggest that certain orders of the Zygomycota may be distinguished on the basis of major sterol. Also, if sterol structure has phylogenetic implications, then orders might be arranged in the order Kickxellales (C_{28} $\Delta^{5,7}$) → Dimargaritales, Zoopagales and Mucorales (C_{28} $\Delta^{5,7,22}$) on the basis of evolution of the predominant and presumably most competent sterol, ergosterol. Although the Entomophthorales would be expected to be more primitive than the above orders based on the predominance of C_{28} Δ^5, it is not apparent from these data that members of the Zygomycota with ergosterol or its precursors as major sterols evolved from this taxon or the Chytridiomycota.

Lipids 32, 1309–1316 (1997).

Ergosterol [**I**, (22*E*, 24*R*)-24-methyl-cholesta-5,7,22-trienol] is considered to be the "fungal sterol," and is typically present at greater than 50% and often up to 85 to 96% of the total sterols in most species of higher fungi, i.e., Ascomycota and Basidiomycota (1). However, there are well-known exceptions to this such as the Uredinales (rust fungi) which produce no ergosterol but have C_{29} Δ^7 and C_{29} $\Delta^{7,24(28)}$ sterols as major sterols, and members of the Taphrinales which produce brassicasterol [(24*R*)-24-methyl-cholesta-5,22-dienol] as the major sterol. On the other hand, the most primitive of the true fungi, i.e. Chytridiomycota, do not produce ergosterol but instead produce either cholesterol (**III**, cholest-5-enol) and/or Δ^5 C_{28} and/or C_{29} monoenes as major sterols (2).

Ergosterol also appears to be the primary sterol of most species of the Zygomycota (1), but this is based on the analyses of relatively few species, mainly from the order Mucorales. However, there are also known exceptions to this such as in *Mortierella alpina,* where there is no ergosterol and where desmosterol (**II**, cholesta-5,24 dienol) is the principal sterol (3), and in *Absidia glauca* and *Mucor hiemalis*, where 22-dihydroergosterol is the major sterol and is accompanied by ergosterol (4). The number of representatives of the various orders and families that have been studied in this regard is insufficient to know whether these are simply exceptions or taxonomic trends that may have phylogenetic implications. Because some of the most primitive fungi have mainly cholesterol or corresponding C_{28} or C_{29} derivatives as the major sterols, and the most advanced fungi have ergosterol as the main sterol, it is reasonable to expect that progressive structural changes from C_{27} Δ^5 to C_{28} $\Delta^{5,7,22}$ sterols might exist within the phylogenetically intermediate phylum Zygomycota. Therefore, fungi representing most orders of the class Zygomycetes, and most families of the order Mucorales, were examined in this study to determine if ergosterol is indeed the principal sterol of the Zygomycota as it currently seems, or if structural differences occur among the orders and families of this phylum that may have phylogenetic implications. In addition, one species of the class Trichomycetes was included in this study.

MATERIALS AND METHODS

Sources of fungi and cultivation of fungi. Fungi analyzed in this study were obtained from various sources as shown in Tables 1 and 2. *Smittium micronatum* was obtained from Dr. R. Lichtwardt (University of Kansas, Lawrence, KS) and *Basidiobolus ranarum* was obtained from Dr. Meredith Blackwell (Louisiana State University, Baton Rouge, LA). They were grown in a 2% glucose, 1% yeast extract medium at 24°C for 6 d unless otherwise noted. Mycelia were harvested by suction filtration, washed with water, dried by lyophilization, and stored at −70°C prior to analysis.

Except as noted elsewhere, the taxonomic placement of the species in this study into families and others is according to Alexopoulos *et al.* (5).

Lipid extraction. Total lipid was extracted from dry cells

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Abbreviations: GLC, gas–liquid chromatography; TMS, trimethylsilyl ether.

TABLE 1
Major Sterols of the Zygomycota[a]

Order/Family/Genus species	I.D. number	Source[b]	Major sterol	Percentage
Dimargaritales				
Dimargaritaceae				
Dimargaris baccillispora	42702	ATCC	Ergosterol	59
Dispira simplex	42701	ATCC	Ergosterol	59
Tieghemiomyces californicus	448.59	CBS	Ergosterol	92
Entomophthorales				
Entomophthoraceae				
Entomophthora destruens	32863	ATCC	24-Methyl cholesterol	92
E. gigantea[b,c]	44588	ATCC	24-Methyl cholesterol	92
E. pyriformis	24419	ATCC	24-Methyl cholesterol	94
Ancylistaceae				
Conidiobolus adiaeretus	12589	ATCC	24-Methyl cholesterol	51
C. bangalorensis[b,c]	16576	ATCC	24-Methyl cholesterol	87
C. obscura	26005	ATCC	24-Methyl cholesterol	94
C. virulenta[d]	2780	NRRL[e]	24-Methyl cholesterol	99
Delacroixia coronatus	10051	ATCC	Cholesterol	100
Basidiobolaceae				
Basidiobolus ranarum		Blackwell[f]	24-Methyl cholesterol/ 24-Methylene-cholesterol	67/33
Glomales				
Glomus caledonius[g]			24-Methyl cholesterol	95
Acaulospora laevis[h]			24 Ethyl cholesterol/cholesterol	80/11
Kickxellales				
Kickxellaceae				
Coemansia erectus	336.87	CBS	22-Dihydroergosterol	91
Martensiomyces pterosporus[d]	2642	NRRL	22-Dihydroergosterol	88
Dipascomyces acuminosporus[d]	2925	NRRL	22-Dihydroergosterol	96
Spiromyces aspiralis[d]	22631	NRRL	22-Dihydroergosterol	92
Mucorales (see Table 2)				
Zoopagales				
Piptocephalidaceae				
Syncephalis spherica	36849	ATCC	Ergosterol/22-dihydroergosterol	49/35

[a]Orders analyzed in this study except for the Mucorales which are in Table 2.
[b]ATCC, American Type Culture Collection (Rockville, MD); CBS, Central Bureau voor Schimmel Cultures (Baarn, The Netherlands); NRRL, Northern Regional Research Laboratory (NCAUR, USDA, ARS, Peoria, IL); Blackwell.
[c]Cultured at 13°C for 6 d.
[d]Cultured at 28°C for 6 d.
[e]Cultures obtained from Gerald Benny, Department of Plant Pathology, University of Florida (Gainesville, FL) *via* the USDA National Regional Research Laboratory (NRRL) (Peoria, IL).
[f]Culture was obtained from Dr. Meredith Blackwell, Department of Plant Biology, Louisiana State University (Baton Rouge, LA).
[g]Data from Beilby and Kidby (Ref. 18).
[h]Data from Beilby (Ref. 19).

(*ca.* 70 mg) using the Bligh and Dyer (6) procedure that was modified according to Kates (7), but with twice the solvent volume-to-tissue dry weight ratio recommended by the latter. All extractions and subsequent manipulation of the samples were conducted at low light intensity.

Sample derivatization and gas–liquid chromatographic analysis (GLC). To obtain the nonsaponifiable lipid, a portion of the total lipid (*ca.* 20 mg) was heated for 2.5 h in 5 mL of 95% ethanol containing 0.5 mL of 33% (wt/vol) KOH (7). The nonsaponifiable lipids were removed from the hydrolysate by washing it three times with 5 mL of hexane. The combined hexane washes were washed twice with 5 mL 3% KOH, twice with 5 mL H_2O, and then brought to dryness under a stream of nitrogen gas.

Sterols in the nonsaponifiable fraction were analyzed by GLC as their trimethylsilylether (TMS) derivatives. Steryl-TMS derivatives were prepared by heating a portion (*ca.* 5 mg) of the nonsaponifiable lipid obtained as described above with 1 mL bis-(trimethylsilyl) trifluoroacetamide/trimethylchlorosilane (99:1, vol/vol) (Supelco Inc., Bellefonte, PA) at 70°C for 25 min. The solvent was evaporated under nitrogen gas and the samples were diluted with hexane prior to analysis by GLC. The gas chromatograph (Hewlett-Packard model 5710; Palo Alto, CA) was equipped with a 25 m × 0.25 mm i.d. fused-silica DB-5MS column (J&W Scientific, Folsom, CA). Injector and detector temperatures were 300°C, and the oven temperature was 270°C isothermal.

GLC–mass spectrometry. GLC–mass spectrometry analysis of the total sterols was performed using a VG 70E high-resolution mass spectrometer (Manchester, United Kingdom)

TABLE 2
Major Sterols of Families of the Mucorales

Family/genus species	I.D. number	Source[a]	Major/minor sterol	Percentage
Absidiaceae				
Circinella angarensis	196	RSA	Ergosterol	84
Absidia spinosa	2401	RSA	Ergosterol/22-dihydroergosterol	79/16
Chaetocladiaceae[b]				
Chaetocladium brefeldii	22825	ATCC	Ergosterol	74
Dichotomocladium hesseltinei[c]	1729	RSA/Benny	Ergosterol/episterol	54/10
D. floridanum[c]	2128	RSA/Benny	Ergosterol	93
Cunninghamellaceae				
Cunninghamella homothalicus	16161	ATCC	Ergosterol/C_{28} tetraene	46/27
Dicranophoraceae				
Syzygites megalocarpus	18025	ATCC	Ergosterol/episterol	53/19
Spordinella umbellata[c]	20824	NRRL	Ergosterol/C_{28} diene #2[d]	70/16
Choanephoraceae				
Choanephora curcubitarum	—	NRRL	Ergosterol/episterol	73/12
Gilbertallaceae				
Gilbertella persicaria	38591	ATCC	Ergosterol	86
Mortierellaceae				
Echinosporangium transversale[c]	3116	NRRL	Desmosterol/C_{28} diene #1[d]	70/26
Mortierella alpina[c]	32222	ATCC	Desmosterol/24-methylene cholesterol	66/14
Mucoraceae				
Parasitella parasiticus[c]	2501	NRRL	Ergosterol/C_{28} diene #2[d]	69/16
Umbelopsis nana[c]	22420	NRRL	Ergosterol/C_{29} $\Delta^{5,7,22}$	80/17
Mycotyphaceae				
Mycotypha microspora[c]	282443	IMI/Benny	Ergosterol	63
Benjaminella youngii[c]	325629	IMI/Benny	Ergosterol/22-dihydroergosterol	71/14
Pilobolaceae				
Pilaira anomala[c]	1998	RSA Benny	Ergosterol/C_{28} tetraene	37/11
Utharomyces epallocaulus	42705	ATCC	Ergosterol/episterol	47/25
Phycomycetaceae				
Phycomyces blakesleeanus	32973	CBS	Ergosterol/episterol	63/21
Radiomycetaceae				
Hesseltinella visculosa[c]	3301	NRRL	Ergosterol	74
Radiomyces mexicanus[c]	1186	RSA/Benny	Ergosterol/C_{28} diene #2[d]	61/20
Sakenaeaceae[e]				
Sigmoidiomycedaceae[e]				
Syncephalastraceae				
Syncephastrum racemosum	8981	ATCC	Ergosterol/22-dihydroergosterol	86/12
Thamnidaceae				
Ellisomyces anormalus[c]	2456	NRRL	Ergosterol	93
Kirkomyces condense[c]	1222	RSA	Ergosterol	78
Helicostylum pulchrum[c]	107.23	CBS	Ergosterol/22-dihydroergosterol	47/46

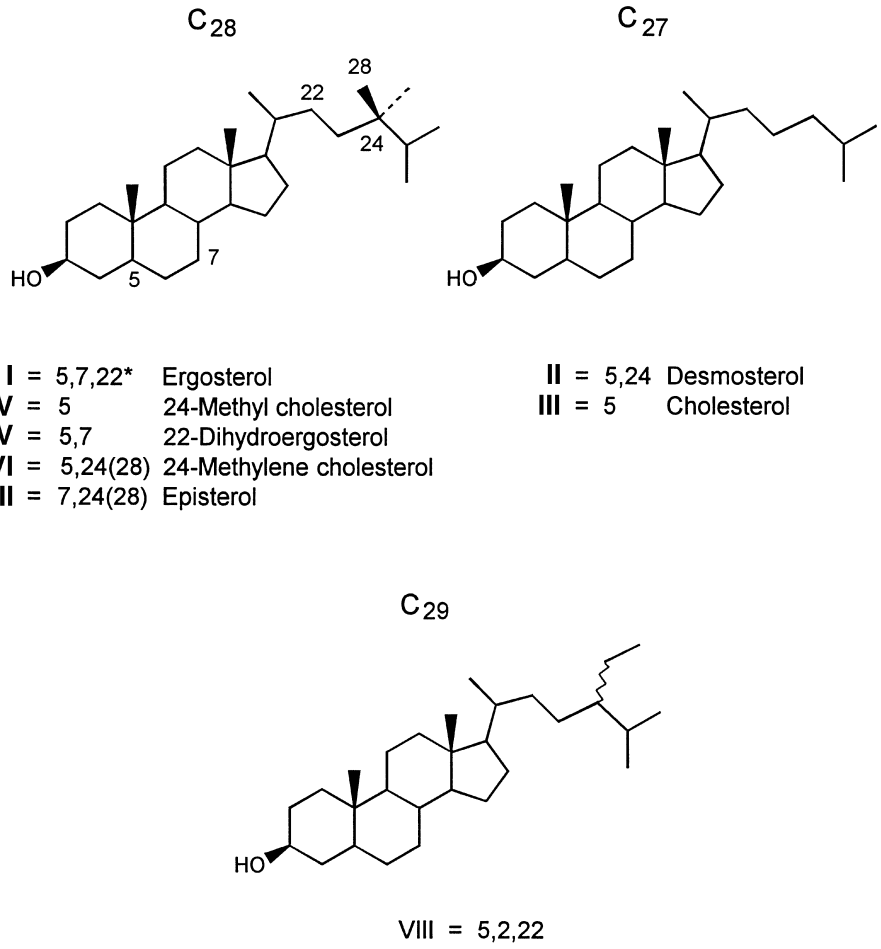
[a]Abbreviations: RSA, Ranco Santa Ana Botanic Gardens; International Mycological Institute (IMI)/Benny. For other abbreviations see Table 1, footnote *b*.
[b]According to Benny and Benjamin (Ref. 13).
[c]Fungi were cultured at 24°C.
[d]See the Results and Discussion section.
[e]Sterols of species from these families have not been analyzed.

linked to a Varian 3700 gas chromatograph (Palo Alto, CA) equipped with a 25 m × 0.25 mm i.d. DB-5MS fused-silica capillary column as the sample inlet, and operated in the electron impact mode at 70 eV with the source temperature at 200°C.

RESULTS AND DISCUSSION

Identification of sterols. The number of sterols detected in the species analyzed in this study ranged from 1 to 18 depending on the species. Only the major sterols of each species are reported here with no more than two sterols per species given. A second major sterol is included if the primary major sterol constitutes less than 80% of the total sterols and another sterol represents 10% or more.

Five different sterols were considered primary major sterols in the fungi of this study according to the above criteria, the most common of which was ergosterol (**I**) (Scheme 1). The mass spectra of the TMS derivative of ergosterol, 24-methyl cholesterol (**IV**, 24-methyl-cholest-5-enol), cholesterol, and desmosterol matched those of authentic standards and are consistent with previously published spectra of these



SCHEME 1

sterols (8) (Table 3). Identification of 22-dihydroergosterol (**V**, 24-methyl cholesta-5,7-dienol) was based on the molecular ion (M^+) at *m/z* 470 and prominent $M^+ - 131$ fragment ion that is characteristic of the TMS derivative of a C_{28} $\Delta^{5,7}$ diene (8).

Several other sterols were detected and included as second major sterols according to the above criteria. The identification of 24-methylene cholesterol [**VI**, 24-methyl cholesta-5,24(28)-dienol] was based on the molecular ion at *m/z* 470 for the TMS derivative, presence of the $M^+ - 129$ and prominent *m/z* 129 which are indicative of the double bond at C-5(6) (8), and prominent fragment ions at *m/z* 296 and 386 for the double bond at C-24(28) (9). Episterol [**VII**, 24-methyl-cholesta-7,24(28)-dienol] was identified on the basis of *m/z* 470 (M^+), 386, and base peak at *m/z* 343. 24-Ethyl-cholesta-5,7,22-trienol (**VIII**) was identified on the basis of m/z 482 (M^+) and major fragment ions corresponding to those of the TMS derivative of ergosterol but 14 mass units higher (Table 3). 22-Dihydroergosterol was detected as a second major sterol in several species (Tables 1 and 2).

Three other sterols were detected as second major sterols, two of which were C_{28} dienes. Only one of the dienes contained a double bond in the C-5(6) position, but location of the second double bond was uncertain (Diene #1). The other diene (Diene #2) did not have a double bond at the C-5(6) position based on the absence of the $M^+ - 129$ fragment ion for the TMS derivative. This sterol was distinguished by the 100% fragment ion at *m/z* 255 and a very prominent molecular ion and is probably 24-methyl cholesta-7,22-dienol (Table 3). The third unidentified sterol was a C_{28} tetraene characterized by *m/z* 466 (M^+), 376, and base peak at 251 suggesting that three of the double bonds are located in the nuclear ring system. Based on the occurrence of known C_{28} sterols with four double bonds (1), the most likely candidate for this sterol has double bonds at the 5,7,9 and 24(28) positions.

Sterols of the Dimargaritales. The sterol composition of representatives from three of the four genera of this monotypic order were analyzed in this study, and ergosterol was the major sterol in each (Table 1). The sterol profiles of *Dimargaris bac-*

TABLE 3
Major Sterols of Fungi of the Phylum Zygomycota[a]

Systematic name (common name)	Symbol	Major fragment ions (*m/z*)
Major sterols		
I. Cholesta-5, 24-dienol (desmosterol)	$C_{27}\ \Delta^{5,24}$	456 (M^+), 441, 372, 366, 351, 343, 327 ($M^+ - 129$) 253, 129, 69 (100%)
II. 24-Methyl-cholesta-5,7,22-trienol (ergosterol)	$C_{28}\ \Delta^{5,7,22}$	468 (M^+), 378, 363 (100%), 337, 253, 69
III. 24-Methyl-cholesta-5,7-dienol (22-dihydroergosterol)	$C_{28}\ \Delta^{5,7}$	470 (M^+), 455, 378, 363 (100%), 337 ($M^+ - 131$), 253
IV. 24-Methyl-cholest-5-enol (24-methyl cholesterol)	$C_{28}\ \Delta^{5}$	472 (M^+), 457, 382, 367, 343 (100%), 261, 255, 213, 129
Minor sterols		
V. 24-Methyl-cholesta-5, 24(28)-dienol (24-methylene cholesterol)	$C_{28}\ \Delta^{5,24(28)}$	470 (M^+), 455, 386, 343, 296, 227, 213, 83 (100%)
VI. 24-Methyl-cholesta-7,24(28)-dienol (episterol)	$C_{28}\ \Delta^{7,24(28)}$	470 (M^+), 455, 386, 343 (100%), 296, 227, 213
VII. 24-Ethyl-cholesta-5,7,22-trienol	$C_{29}\ \Delta^{5,7,22}$	482 (M^+), 392, 377 (100%), 351, 253
	C_{28} Diene #1	470 (M^+), 455 ($M^+ - 15$), 380, 365, 341 ($M^+ - 129$), 271, 129 (100%)
	C_{28} Diene #2	470 (M^+), 455 ($M^+ - 15$), 427, 372, 365, 343, 318, 255 (100%), 229, 213, 75
	C_{28} Tetraene	466 (M^+), 451 ($M^+ - 15$), 376, 361, 251 (100%)

[a]See Scheme 1 for sterol structures.

cillispora and *Dispiria simplex* were essentially the same, with episterol accompanying ergosterol at 19 and 16% of the total sterols, respectively (Table 1). *Tieghemiomyces californicus* contained 92% ergosterol.

Sterols of the Entomophthorales. The sterol composition of four genera representing three of the six families was determined in this study, i.e., three *Entomophthora* species of the Entomophthoraceae, and four *Conidiobolus* species and one *Delacroixia* species of the Ancylistaceae, and *B. ranarum* of the Basidiobolaceae. *Entomophthora* and *Conidiobolus* species were distinct from those in the other Zycomycota orders with 24-methyl cholesterol as the major sterol at 87 to 94% of the total sterols in six of the seven species analyzed. *Conidiobolus adiaeretus* and *B. ranarum* contained only 51 and 70% of this sterol, respectively (Table 1). The latter species contained 24-methylene cholesterol as the second major sterol. Distinguishing *Conidiobolus* from *Entomophthora* species on the basis of morphology has proven difficult and has required the use of nuclear characters to separate them (10), and representatives of the two genera could not be distinguished on the basis of sterol composition.

On the other hand, *Delacroixia coronatus*, formally *C. coronatus*, contained cholesterol as the single sterol detected. More species must be analyzed to determine if this departure from the $C_{28}\ \Delta^5$ structure is an exception or a trend within the Ancylistaceae family.

Sterols of the Kickxellales. Starr *et al.* (11) reported that cholesterol was the only sterol detected in *Dipsacomycetes acuminosporus* and *Linderina pennispora*. However, Weete and Laseter (12) did not detect cholesterol or ergosterol in spores of *L. pennispora* but instead reported a C_{28} diene among two other sterols detected. In this study, the major sterol of *D. acuminosporus* was the C_{28} diene 22-dihydroergosterol at 96% of the total sterols (Table 1). This sterol may be characteristic as the principal sterol of this order since it was also predominant in *Coemansia erectus* and *Martensiomyces pterosporus* (Table 1).

Sterols of the Zoopagales. Only one species from one of the four families of this order was included in this study, i.e., *Syncephalis spherica* of the Piptocephalidaceae, which contained 49% ergosterol and 35% 22-dihydroergosterol (Table 1).

Sterols of the Mucorales. Twenty-five species belonging to the order Mucorales, representing 14 of the 16 families (5,13) were examined in this study for sterol composition. With one exception, the major sterol of the species from the various Mucorales families was ergosterol (Table 2). In *Heliocostylum pulchrum* (Thrammidiaceae), 22-dihydroergosterol occurred in about equal proportions with ergosterol at 46% of the total sterols, whereas the other two species from this family analyzed in this study contained 78% or more ergosterol (Table 2). In this study, *Absidia spinosa* contained 79% ergosterol and 16% 22-dihydroergosterol, but McCorkindale *et al.* (4) reported that *A. glauca* contained 83% of the latter sterol and 12% of the former.

The exception noted above was the Mortierellaceae where ergosterol was absent from *Mortierella alpina* (IS-4) and desmosterol was the major sterol (3). We have found this to be true for another strain of this species (Table 2). Desmosterol may be characteristic of this family because *Echinosporangium transversale*, also of the Mortierellaceace, contained

desmosterol as the major sterol, and no ergosterol was detected (Table 2). This is unusual because with few exceptions C_{28} sterols tend to be predominant in fungi that are phylogenetically more advanced than those in the phylum Chytridiomycota (1). Although a C_{27} sterol is the primary major sterol of *M. alpina* and *E. transversale,* they are capable of carrying out the alkylation at C-24 as indicated by the presence of C_{28} sterols such C_{28} $\Delta^{5,?}$ in *E. transversale* and 24-methylene cholesterol in *M. alpina* as second major sterols (Table 2), and several other C_{28} alkylsterols in each species. These results are consistent with the taxonomic uncertainty of the Mortierellaceae (14) and reinforce the question of whether this family should be included in the Mucorales.

In other studies with Mucorales fungi, ergosterol has been detected as the major sterol in *Rhizopus arrhizus* (15) and *Mucor* species (16,17). However, there may be exceptions such as *M. hiemalis,* where 22-dihydroergosterol was the principal sterol (4), but Mucoraceae is a large family and many more species representing such genera should be analyzed before definitive conclusions can be drawn.

The only families of the order Mucorales that remain to be studied with respect to sterol composition are the Saksenacaceae and Sigmoidiomycetaceae which are single-genus and two-genus families, respectively (5).

Other orders. To our knowledge, representatives of the Endoglonales have not been studied with respect to sterol composition, but the vesicular-arbuscular mycorrhizal fungi *Acaulospora laevis* and *Glomus caledonius* of the order Glomales reportedly produce cholesterol and 24-methyl cholesterol as major sterols (18), and 24-ethyl cholesterol along with 24-methyl cholesterol and cholesterol, respectively, but no ergosterol (19).

Sterols of the Trichomycetes. Because of the possible close affinity of members of the order Harpellales with those of the Kickxellales and of the possible uniqueness of the major sterols in the latter group, i.e., 22-dihydroergosterol (Table 1), *Smittium micronatum* was included in this study. Desmosterol was reported in all but two of 14 *Smittium* isolates, and cholesterol, ergosterol, and a C_{28} diene were detected in some of them (11). In this study, the principal sterol of *S. micronatum* was ergosterol at 63% of the total sterols; 22-dihydroergosterol, as a relatively minor component at only 6%, may be the M^+ 470 component (TMS derivative) detected by Starr *et al.* (11). Other sterols detected in this species were a C_{28} monoene (Δ^7 or Δ^8) (7%), a C_{28} diene (11%), and a C_{30} diene (13%), none of which had a double bond in the C-5 position. Neither cholesterol nor desmosterol was detected in this species.

As we have suggested previously (1), it is unlikely that sterols alone are useful taxonomic characters for distinguishing fungi at the species or even genus levels of classification. However, the results of this study indicate that the designation of ergosterol as the "fungal sterol" does not apply to all orders of the phylum Zygomycota, i.e., members of some orders have primary major sterols other than ergosterol. Although relatively few zygomycetous fungi have been studied in this regard, the results of this study indicate that two of the orders of this phylum, i.e., Entomophthorales and Kickxellales, might be distinguished from one another, and from the orders Dimargaritales, Mucorales and Zoopagales, on the basis of their primary major sterol, i.e., 24-methyl cholesterol and 22-dihydroergosterol, respectively, opposed to ergosterol.

There appear to be no trends with respect to the second major sterols, except possibly that species in the Dimargaritales, Entomophthorales, and Kickxellales exhibited less tendency to accumulate such sterols than members of the other orders. Generally, in cases where the primary major sterol was less than 80%, no other sterol accumulated to greater than 10% of the total sterols in these taxa. Exceptions to this were *Syncephalis spherica* of the Zoopagales and many species of the Mucorales (Tables 1 and 2). In the latter order, 16 of the 25 species contained second major sterols, ranging from 10 to 46% of the total sterols, but there was no obvious pattern to their distribution among the families (Table 2).

Cholesterol is the principal sterol in some species of the most primitive fungi (e.g., Chytridiomycota) as well as in the evolutionarily most advanced organisms (e.g., mammals). It sometimes functions in artificial membranes, and it supports the growth of a sterol-requiring prokaryotic microbe better than others tested (20), including ergosterol (21). Nevertheless, ergosterol is the main sterol of fungi at the top of the fungal evolutionary hierarchy, Ascomycota and Basidiomycota; and cholesterol is absent in these taxa (1), suggesting that this sterol may perform a specific function in fungi related to its structure or offer some advantage that is unique to these fungi. Ergosterol satisfies the sterol requirement for the growth of yeast cultured under anaerobic conditions better than cholesterol and others tested (22). Parks and Casey (23) suggested that ergosterol may be the preferred sterol because it serves as a consensus sterol in satisfying multiple functions.

Although only a relatively few species of chytrids have been analyzed in this regard, there is no apparent pattern to the distribution of sterols among the four orders represented, i.e., cholesterol is the major sterol in a species of three of the four orders whereas other species of these orders have other dominant sterols in the absence of cholesterol (2). This is consistent with the contention that there is considerable phylogenetic divergence among the chytrids (24). Because members of the Blastocladiales (*Blastocladiella emersonii* and *Allomyces macrogynus*) have cholesterol as the major sterol (2), and they are believed to be among the more primitive of the chytrids, then this sterol may be considered the most primitive sterol, at least in eucaryotes, and alkylation at C-24 might be considered to be associated with the move toward the evolution of more advanced fungi. One of the structural differences between cholesterol and ergosterol is the presence of the 24-methyl group (Scheme 1). Alkylation at C-24 occurs prior to the demethylation of lanosterol at C-4 and C-14 during the formation of ergosterol in at least the few higher fungi and zygomycetes that have been studied in this regard (25). Although whether alkylation occurs before or after (as in yeast, Ref. 25) the demethylation of lanosterol in the chytrids

has not been tested, some species of this taxa can carry out this reaction as indicated by the presence of 24-alkyl sterols.

Although it appears as a second major sterol in Hyphochytridiomycetes and the chytrid *Monoblepharella* sp. (2), 24-methyl cholesterol was found as the major sterol only in species belonging to the Entomophthorales, with the exception of *D. coronatus* which contained cholesterol as the principal sterol. As in the entomophthoralean fungi, 24-methyl cholesterol was the major sterol of *B. ranarum* but, unlike other Entomophthorales species or any other fungus of this study, a significant second major sterol of this species was 24-methylene cholesterol which is common in the Oomycota (4) and *Rhizophydium sphaerotheca* (Chytridales) (2). However, molecular analysis placed *B. ranarum,* which is considered to be an entomophthorean fungus based on morphological characteristics (5), in a cluster with the chytrids *Chytridium*, *Spizellomyces* and *Neocallimastrix* rather than the cluster with *Entomophthora, Conidiobolus* and *Zoophthora* (24). Therefore, the taxonomic placement of *B. ranarum* in either the Chytridiomycota or Entomophthorales cannot be resolved on the basis of major sterol composition. Cavalier-Smith (26) considered the entomophthoralean fungi to be ancestral zygomycetes derived from the chytrids. The sterol composition is consistent with this view if we consider 24-methyl cholesterol to be a more primitive sterol than ergosterol but not unequivocal (Fig. 1).

The other structural features that distinguish ergosterol from cholesterol are the double bonds at C-7 and C-22. Since $\Delta^8 \rightarrow \Delta^7 \rightarrow \Delta^{5,7} \rightarrow \Delta^5$ is considered to be the reaction sequence in the sterol ring system in the latter stages of cholesterol and ergosterol formation (25), it is likely that the first step toward ergosterol biosynthesis arose as a defect in the formation of 24-methyl cholesterol, i.e. loss of the ability to reduce the double bond at C-7, thus giving rise to 22-dihydroergosterol (C_{28} $\Delta^{5,7}$). If this sterol occurs throughout the order Kickxellales as may be the case based on the results of this study, then the occurrence of 22-dihydroergosterol as the principal sterol may have evolutionary implications. This suggests that the Kickxellales are among the most primitive Zygomycetes, but whether the evolutionary process occurred directly from a chytrid, or *via* the Entomophthorales, cannot be disinguished from this data (Fig. 1).

The next step leading to the formation of ergosterol would be the capacity to introduce a double bond at C-22. If the evolution of fungi is associated with the ability to introduce a double bond at this position, and 7-dihydroergosterol is not a major sterol, then the Dimargaritales, Mucorales, and Zoopagales should be phylogenetically more advanced than the Kickxellales on the basis of sterol composition. Although ongoing RNA sequencing research may shed some light on this in the near future, unfortunately our knowledge of the evolutionary relationships among the zygomycetous fungi at the order level of classification is insufficient at this time to be able to arrange them in a definitive sequence from the most primitive to the most advanced.

Although several functions for ergosterol have been detected in yeast (27), the precise nature of these functions is not known, except for their role in membranes (28). Two of the functions are the bulk or membrane role, which has the lesser structural specificity and requires more sterol, and the sparking role, which has the greater structural specificity and requires the lesser amount, i.e., requirement for the double bond at C-5(6) (27). Based on structure–function studies whereby genes for essentially all of the steps in the postcyclization stage of ergosterol biosynthesis have been inactivated and tested individually for their essentiality, and the cells with the lesions are viable and able to grow, it is difficult to rationalize the driving force for the evolution of the ergosterol structure. However, it has been shown recently that the further back toward lanosterol that inactivation of a step in ergosterol biosynthesis occurs, at least in *Saccharomyces cerevisiae*, the less competitive the strain containing the lesion is with the wild type (29).

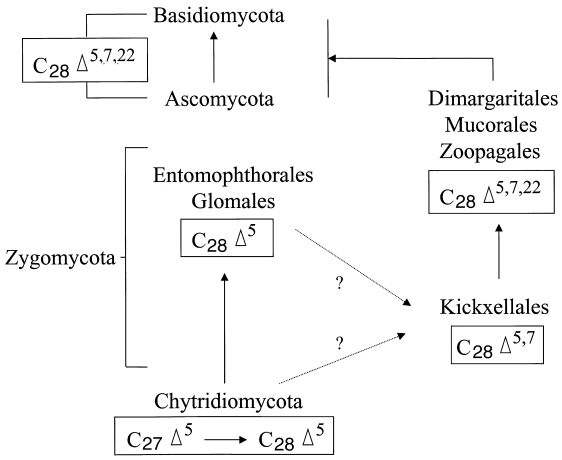


FIG. 1. Schematic showing the possible evolution of sterols from cholesterol to ergosterol from the primitive phylum Chytridiomycota through the phylum Zygomycota to the Ascomycota and Basidiomycota.

Bloch (30) speculated that evolutionary pressures were responsible for driving structural modifying events (i.e., demethylation at C-4 and C-14) that led to improvement in the functional competence of sterols, i.e., lanosterol → cholesterol. He also pointed out that there was an increase in the ability of sterol biosynthesis intermediates differing by decreasing degrees of methylation at C-4 and C-14 to function in artificial membranes and showed that the ability of these sterols to support the growth of a sterol-requiring microbe was correlated to the progressive loss of methyl groups at these positions. In this context, it is tempting to speculate that retention of the double bond at C-7 and introduction of the double bond at C-22, which lead to ergosterol formation, may have conferred a competitive advantage to the species acquiring these modifications and therefore played a role in the evolution of these fungi. However, based on structure–function studies, double bonds at these positions seem to be the least important from the standpoint of function in supporting growth (yeast) (29).

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Sterol Utilization and Metabolism by *Heliothis zea*

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ABSTRACT: *Heliothis zea* (corn earworm), an insect that fails to synthesize sterols *de novo*, was reared on an artificial diet treated with 18 different sterol supplements. Larvae did not develop on a sterol-less medium. Δ^5-Sterols with a hydrogen atom, a methylene group, an *E*- or *Z*-ethylidene group, or an α- or β-ethyl group (cholesterol, ostreasterol, isofucosterol, fucosterol, sitosterol, and clionasterol, respectively) at position C-24, and Δ^5-sterols doubly substituted in the side chain at C-24 with an α-ethyl group and at C-22 with a double bond (stigmasterol) supported normal larval growth to late-sixth instar (prepupal: maturity). The major sterol isolated from each of these sterol treatments was cholesterol, suggesting that *H. zea* operates a typical 24-dealkylation pathway. The sterol requirement of *H. zea* could not be met satisfactorily by derivatives of 3β-cholestanol with a 9β,19-cyclopropyl group, *gem* dimethyl group at C-4, a $\Delta^{5,7}$-bond or Δ^8-bond, or by side-chain modified sterols that possessed a $\Delta^{25(27)}$-24β-ethyl group, $\Delta^{23(24)}$-24-methyl group or 24-ethyl group, or $\Delta^{24(25)}$-24-methyl or 24-ethyl group. The major sterol recovered from the larvae (albeit developmentally arrested larvae) treated with a nonutilizable sterol was the test compound. Sterol absorption was related to the degree of sterol utilization. The most effective sterols absorbed by the insect ranged from 27 to 66 μg per insect, whereas the least effective sterols absorbed by the insect ranged from 0.6 to 6 μg per insect. Competition experiments using different proportions of cholesterol and 24-dihydrolanosterol (from 9:1 to 1:9 mixtures) indicated that abnormal development of *H. zea* may be induced on less than a 1 to 1 mixture of utilizable (cholesterol) to nonutilizable (24-dihydrolanosterol) sterols. The results demonstrate new structural requirements for sterol utilization and metabolism by insects, particularly with respect to the position of double bonds in the side chain and functionalization in the nucleus. The novel sterol specificities observed in this study appear to be associated with the dual role of sterols as membrane inserts (nonmetabolic) and as precursors to the ecdysteroids (metabolic).

Lipids 32, 1317–1323 (1997).

Insects, unlike plants, are unable to synthesize sterols *de novo*, and therefore acquire sterols from feeding on plants and/or from symbionts (1,2). The resulting sterol ecology that evolved in insect–plant interactions has contributed to the design of novel insect control technologies based on modification of the phytosterol pathway (3). Most insects can be reared on sterols that share the same structural characteristics of cholesterol (cholest-5-en-3β-ol), viz., a 3β-hydroxyl group, a planar tetracyclic nucleus, and an intact side chain of 8 to 10 carbon atoms (1–3). Phytophagous insects may absorb and utilize a variety of dietary sterols, structurally similar to cholesterol as tissue sterols (1,2). In many insects, C-24 dealkylation of phytosterols is a critical step in ecdysteroid production (3). It can be assumed that several possible intermediates of sitosterol production, including the C-24 dealkylation reaction of phytosterols, should satisfy the sterol requirement of insects. The pathway of phytosterol C-24 dealkylation has been shown to proceed from 24-ethylcholesterol to cholesterol in four major steps: (i) dehydrogenation of the C-24 ethyl group; (ii) formation of a C-24(28)-epoxide; (iii) deethylation of the C-24(28)-epoxide; and (iv) reduction of the 24,25-double bond (Fig. 1) (3,4). Interruption of this pathway by inhibitors designed to interfere with steps ii and iv has been found to prevent ecdysone synthesis (step v, Fig. 1) and impair normal larval growth and development (2–4).

The objectives of the present study were to establish the structural requirements of sterols in the C-24 dealkylation pathway and to ascertain whether the nutritional requirement for a sitosterol-type sterol by *Heliothis zea* (=*Helicoverpa zea*) was absolute. To this end, various sterols synthesized by plants were fed to *H. zea* singly and in a sparing (partly replace) capacity. As first hypothesized by Clark and Bloch (5), underlying this approach is the recognition that sterols play a dual role in insects: as a membrane insert (nonmetabolic) and as a precursor to the ecdysteroids, molting hormones, (metabolic). Δ^5-Phytosterols such as sitosterol, which are end products of plant metabolism (6), may, after conversion to cholesterol, perform both sterol functions in insects (1,2). On the other hand, sterols which are intermediates in phytosterol turnover, such as 24-ethyldesmosterol (6), may be capable of only partly replacing sitosterol in *H. zea* , because the insect cannot transform the sterol. Phytosterol intermediates might be able to perform only one of the essential sterol functions (e.g., architectural), and thereby must spare cholesterol (metabolic) for the insect to grow. However, for cholesterol to be sparred there must be a source of cholesterol available for ecdysteroid production. When cholesterol is not available and the dietary phytosterol cannot undergo metabolism to cholesterol

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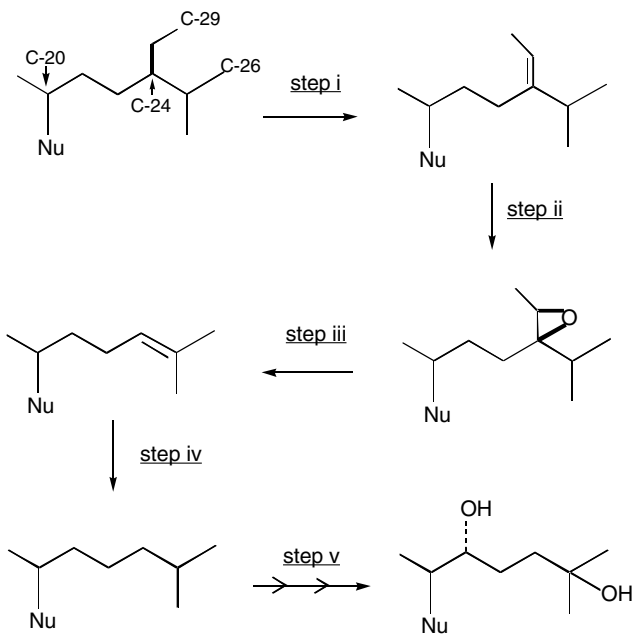

FIG. 1. Hypothetical 24-dealkylation pathway in *Heliothis zea*: a 24-ethylsterol is converted to a Δ^5-24-desalkylsterol in four steps, after which the side chain of the 24-desalkyl sterol is oxidized at C-22 and C-24 to form the side chain of ecdysone (step v involves many steps including oxidation of the side chain and nucleus). See text for further details.

by the insect, then the nutritional sterol is nonutilizable and larval death should ensue. Ritter (7–10), studying sterol structure–activity relationships in *H. zea,* has shown that the type and amount of sterol available to the insect diet influence growth and development. In our comparative studies on metabolism of 4-desmethyl, 4-monomethyl and 4,4-dimethyl sterols fed to *Manduca sexta*, we found that sterol utilization was associated with the level of phytosterol in the medium preparation and the degree of sterol metabolism by the insect (11).

We describe experiments herein designed to shed light on utilization and metabolism of dietary phytosterols by *H. zea*. Our results indicate a heretofore unrecognized sterol specificity in the type of sterols that may serve as membrane inserts and/or as precursors of ecdysteroid production. We chose to study *H. zea* for three reasons: (i) a relatively sterol-free diet has been designed to grow the insect (7); (ii) there has been a series of sterol structure–function studies performed on the insect (7–10); and (iii) *H. zea* is a major pest of corn, causing substantial crop damage annually. *Heliothis zea* has been shown to convert sitosterol to cholesterol (10) and to synthesize ecdysone and 20-hydroxyecdysone (12), suggesting that *H. zea* operates the sitosterol-to-ecdysteroid pathway commonly found in insects (3).

MATERIALS AND METHODS

Culturing of H. zea. Eggs were originally obtained from the Department of Entomology, University of Illinois (Urbana, IL). The stock insects were reared at the United State Department of Agriculture laboratory in Peoria, IL, using sterile procedures on a pinto bean-based diet. Moths were fed 10% sucrose (13). Cultures were maintained at 27 ± 1°C, at 40 ± 10% relative humidity on a 14:10 light–dark photoperiod (13). The artificial diet of Ritter and Nes (7) was used to rear the insects on different sterol supplements. The experimental diet contained Bacto agar, which is known to contain trace contamination of cholesterol (14), otherwise the experimental diet was sterol-free. Sterols were solubilized in acetone. Aliquots of the solutions were added to a warm sterol-free, still-liquid diet, the material mixed thoroughly with the diet using a stir plate, and the organic solvent allowed to evaporate. To each of twenty 35-mL plastic cups was added 1 mg of sterol (equivalent to 200 ppm) and 1 insect at the first instar stage of development. The larvae were allowed to grow and mature for 20 d. The fresh weight and length of 20-d larvae were recorded. Ritter and Nes (7) found that by day 20, *H. zea* larvae are in the final stage of larval development (sixth instar), after which time the larvae may pupate and develop into the moth form. In some sterol treatments, we allowed the larvae to pupate and develop further. We observed that neonate larvae failed to molt to the second instar when sterol was absent from the diet, but as also observed by Ritter and Nes (7), some of these insects may survive for longer periods, presumably due to sparing of egg cholesterol. The minimal dietary concentration of cholesterol necessary for larvae to grow and pupate was found by Ritter and Nes (7) to be 0.01% of the experimental diet. We used 0.02% (200 ppm) to ensure that a nonlimiting amount of the test sterol was available in the experimental diet and growth rates would be maximized.

Sterol source and analysis. Sterols were obtained from our sterol collection (6,15,16, and references cited therein) or synthesized from appropriate sterol substrates that were from the sterol collection. Sterol isolation, purification, quantitation, and identification by chromatographic and spectral methods were as described in our earlier papers (6,15). All sterols were greater than 99% pure, except the $\Delta^{23(24)}$-24-alkylsterols, which were *ca.* a 1-to-1 mixture of *E*- and *Z*-23,24-double bond isomers. Sterols recovered from the nonsaponifiable lipid fraction were analyzed by gas–liquid chromatography–mass spectrometry (6,16) and found to contain long-chain fatty alcohols (17). These fatty alcohols may comigrate with sterols during thin-layer chromatography and interfere with sterol quantitation, particularly that of cholesterol. Therefore, in order to confirm the identity and amount of cholesterol in each insect, an aliquot of the nonsaponifiable lipid fraction was injected into a reversed-phase high-performance liquid chromatograph. The fraction migrating with cholesterol was eluted from the column (in some cases this sample was subfractionated on a second high-performance liquid chromatography column, cf. Ref. 6) and examined by gas–liquid chromatography–mass spectrometry. An authentic specimen of 24,28-fucosterol epoxide was supplied to us by Dr. James Svoboda (United States Department of Agriculture/Agricultural Research Service, Beltsville, MD) for use as a chromatographic standard. Chromatographic

and spectral data of sterols isolated from the larvae matched authentic specimens available to us from earlier studies (4,6,15).

RESULTS AND DISCUSSION

Heliothis zea was found to utilize 7 of 18 naturally occurring phytosterols for growth (Table 1). Many of the phytosterols which are nonutilizable occur in corn plants (6). The mode of action of these sterols on growth and maturation was found to be related to their degree of absorption and metabolism. The corn earworm fed ostreasterol, fucosterol, isofucosterol, sitosterol, clionasterol, and stigmasterol grew and developed normally in the same manner as those reared on cholesterol (Table 1). The utilizable sterols (Fig. 2) were absorbed to similar levels (27 to 71 μg per insect), and converted to cholesterol in high yield by the insect. There was no evidence for fucosterol 24,28-epoxide in the chromatograms of larval sterols, suggesting rapid turnover of the polar intermediates during C-24 dealkylation. From these results, we conclude that *H. zea* operates a typical C-24 alkylation pathway that acts on 24-alkyl Δ^5-sterols. The hypothetical pathway operates as follows: sitosterol conversion to fucosterol; fucosterol conversion to fucosterol 24,28-epoxide; fucosterol 24,28-epoxide conversion to desmosterol; and desmosterol conversion to cholesterol (Fig. 1). Cholesterol is then presumably converted to ecdysone.

The amount of tissue cholesterol that is necessary to support growth is not clear. We examined the influence of cholesterol mixed in different proportions with 24-dihydrolanosterol, (utilizable and nonutilizable sterols, respectively) (Table 2). Insect growth was found to be impaired on a 30:70 mixture and completely inhibited on a 10:90 mixture of cholesterol to 24-dihydrolanosterol (Table 2). Adequate amounts of cholesterol are absorbed by larvae from a 50:50 mixture of cholesterol to 24-dihydrolanosterol to promote growth. The level of cholesterol in these insects represents *ca.* 18 μg cholesterol per insect. The 24-dihydrolanosterol absorbed by the insects is not harmful to growth, and apparently does not compete in a negative manner with cholesterol as a precursor to ecdysteroid production. The amount of cholesterol in the 10:90 cholesterol/24-dihydrolanosterol treatment absorbed by the insect apparently falls below the threshold value necessary to promote growth. Thus, we assume the lower limit of utilizable sterol(s) to promote growth and maturation must be at or below 18 μg per insect. This limit is much different from that for other insects, such as for aphids and the tobacco hornworm (11,17), which are very different-sized insects. The inability of 24-dihydrolanosterol to replace cholesterol as a growth supplement indicates that one or more sterol features on the 24-dihydrolanosterol constitution are harmful to activity.

Larvae fed cholest-8-enol, 24-dehydropollinastanol, 24-methyl cholesta-5,23-dienol, 24-ethyl cholesta-5,23-dienol,

TABLE 1
Effect of Sterols on Growth and Metabolism by *Heliothis zea*

Sterol supplement	Entry number[a]	Growth response[b]	Instar reached by day 20	Total sterol (mg/insect)	Sterol composition (as % total sterol)[c]
Utilizable sterols					
Cholesterol	1	100	6	56	Cholesterol
Ostreasterol	2	100	6	59	Ts/cholesterol (16:84)
Fucosterol	4	100	6	71	Ts/cholesterol (10:90)
Isofucosterol	3	100	6	52	Ts/desmosterol/cholesterol (8:14:78)
Sitosterol	5	100	6	66	Ts/cholesterol (20:80)
Clionasterol	6	100	6	43	Ts/cholesterol (14:84)
Stigmasterol	7	100	6	27	Ts/desmosterol/cholesterol (15:1:84)
Nonutilizable sterols					
Cholest-8-enol	13	5	3	n.d.	n.d.
24-Dehydropollinastanol	14	5	3	0.6	Ts/cholesterol (86:14)
24-Methyl cholesta-5,23-dienol	10	50	5	6	Ts/cholesterol (80:20)
24-Ethyl cholesta-5,23-dienol	12	20	3	3	Ts/cholesterol (86:14)
24-Methyl cholesta-5,24-dienol	9	5	3	1	Ts/cholesterol (65:35)
24-Ethyl cholesta-5,24-dienol	11	10	3	n.d.	n.d.
Clereosterol	8	20	3	3	Ts/cholesterol(80:20)
Ergosterol	15	30	3	5	Ts/cholesta-5,7-dienol/cholesterol (36:41:23)
Cycloartenol	16	5	3	5	n.d.
Lanosterol	17	5	3	n.d.	n.d.
24-Dihydrolanosterol	18	5	3	n.d.	n.d.

[a]Structures of sterols are reported in Figure 2.

[b]Growth on cholesterol produced larva that at 20 d weighed, on average, 323 mg and possessed a length of 30 mm. Generally 16 of 20 insects survived on cholesterol to pupate and develop into moth form. The growth response on test sterols is relative to the growth (larval length) on cholesterol which is normalized to 100%. 24-Methyl cholesta-5,23-dienol was found to develop into pupae and into adult forms. However, these insects exhibited congenital deformities. Insects in the nonutilizable category generally weighed less than 100 mg per insect, and their length ranged from 1.5 to 15 mm, with 6 to 12 insects alive at day 20.

[c]Sterols isolated from insect tissues, after the gut was purged of its contents, were analyzed by reversed-phase high-performance (HPLC) (6) and gas–liquid chromatography–mass spectrometry (GLC–MS) (6); n.d., not determined; and Ts, test sterol.

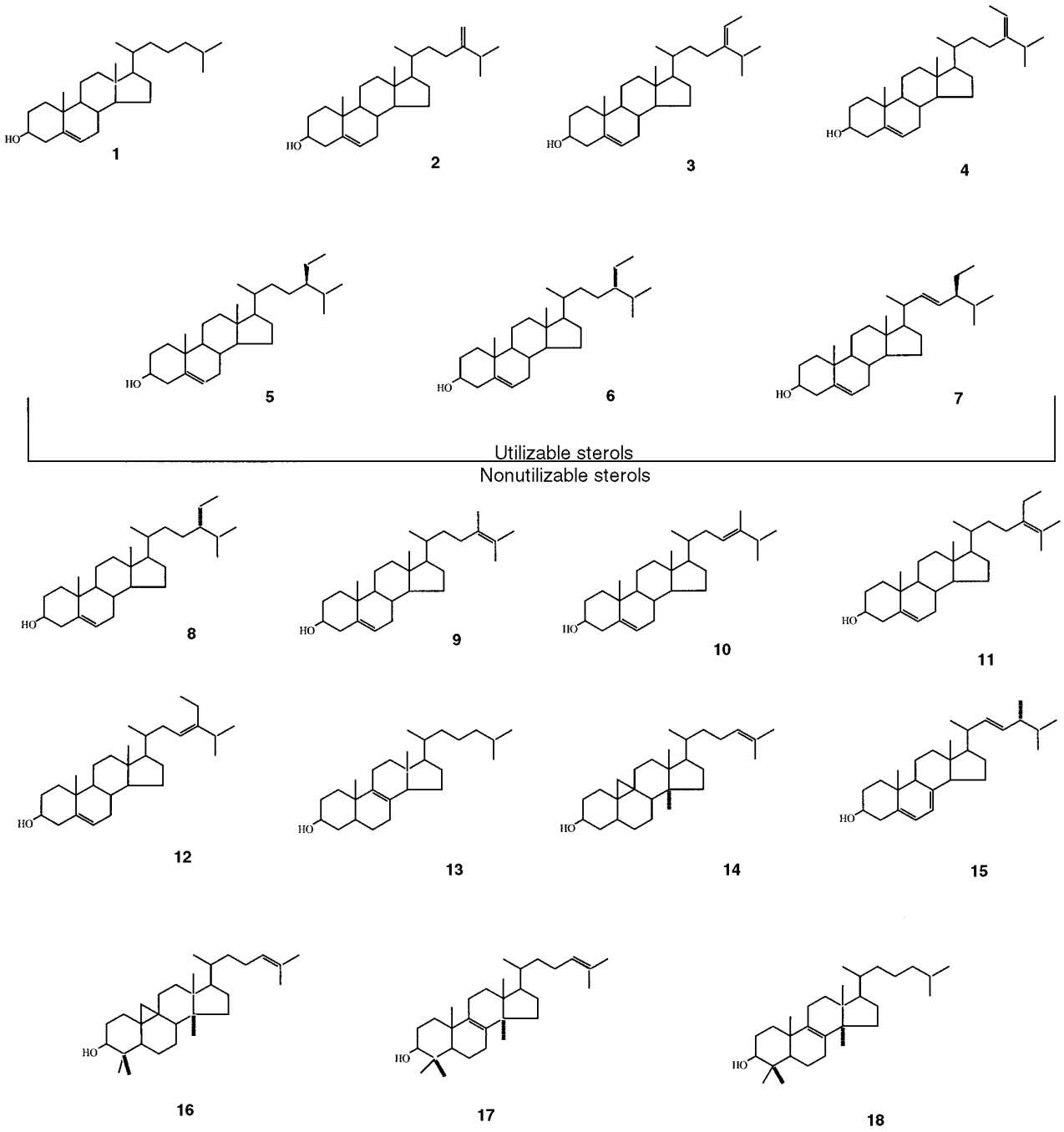

FIG. 2. Structures of sterols fed to *Heliothis zea,* trivial names and (systematic names): (1) cholesterol (cholest-5-en-3β-ol); (2) ostreasterol [cholesta-5,24(28)-dien-3β-ol]; (3) isofucosterol [(24*Z*)-24-ethylidenecholesta-5,24(28)*Z*-dien-3β-ol; (4) fucosterol (24*E*)-24-ethylidene cholesta-5,24(28)*E*-dien-3β-ol; (5) sitosterol [24(*R*,α)-ethylcholest-5-en-3β-ol]; (6) clionasterol [24(*S*,β)-ethylcholest-5-en-3β-ol]; (7) stigmasterol [24(*S*,α)-ethylcholesta-5,*22E*-dien-3β-ol]; (8) clereosterol [24*R*,β)-ethylcholesta-5,25(27)-dien-3β-ol]; (9) 24-methyldesmosterol (24-methylcholesta-5,24-dien-3β-ol); (10) no trivial name (24-methyl cholesta-5,23-dien-3β-ol); (11) 24-ethyldesmosterol (24-ethylcholesta-5,24-dien-3β-ol); (12) no trivial name (24-ethylcholesta-5,23-dien-3β-ol); (13) no trivial name (cholest-8-en-3β-ol); (14) 24-dehydropollinastanol (14α-methyl-9β,19-cyclo-5α-cholesta-8,24-dien-3β-ol); (15) ergosterol [24(*R*,β)-methylcholesta-5,7,22*E*-trien-3β-ol]; (16) cycloartenol (4,4,14-trimethyl-9β,19-cyclo-5α-cholesta-8,24-dien-3β-ol); (17) lanosterol (4,4,14-trimethyl-5α-cholesta-8,24-dien-3β-ol); (18) 24-dihydrolanosterol (4,4,14-trimethyl-5α-cholest-8-en-3β-ol). The sterol numbering system that we prefer to use is based on the biosynthetic side-chain rule discussed in Reference 6 (and references cited therein).

24-methyl cholesta-5,24-dienol, 24-ethyl cholesta-5,24-dienol, clereosterol, ergosterol, cycloartenol, lanosterol, and 24-dihydrolanosterol (Fig. 2) failed to grow normally. These 11 compounds may be considered nonutilizable sterols (Table 1). Larvae from treatments with the nonutilizable sterols either died by day 20 or underwent delayed growth and pupation. The resulting pupae were found to possess aberrant forms, which after development into moth forms exhibited

TABLE 2
***Heliothis zea* Utilization of 24-Dihydrolanosterol Sparred with Cholesterol**

Sterol mixture (ratio)	Entry number[a]	Growth response[b]	Instar reached by day 20	Total sterol (μg/insect)	Sterol composition (as % total sterol)[c]
Cholesterol (100%)	1	100	6	56	Cholesterol (100%)
Cholesterol/24-dihydrolanosterol (90:10)	1:18	100	6	45	Cholesterol/24-dihydrolanosterol (93:7)
Cholesterol/24-dihydrolanosterol (70:30)	1:18	100	6	36	Cholesterol/24-dihydrolanosterol (88:12)
Cholesterol/24-dihydrolanosterol (50:50)	1:18	70	6	25	Cholesterol/24-dihydrolanosterol (75:25)
Cholesterol/24-dihydrolanosterol (30:70)	1:18	30	3	12	Cholesterol/24-dihydrolanosterol (50:50)
Cholesterol/24-dihydrolanosterol (10:90)	1:18	10	3	n.d.	n.d.

[a]Structures of sterols are reported in Figure 2.

[b]Growth on cholesterol produced larva that at day 20 weighed, on average, 323 mg and possessed a length of 30 mm. Generally 16 of 20 insects survived to pupate and developed into moth form. The growth response on test sterol mixtures is relative to the growth on cholesterol (see Table 1) which is normalized to 100%.

[c]Sterols isolated from insect tissues, after the gut was purged of its contents, were analyzed by reversed-phase-HPLC (6) and GLC–MS (6); n.d., not determined. See Table 1 for abbreviations.

congenital deformities. Similar observations were made by Ritter (10), studying *H. zea* fed $\Delta^{5,7}$-sterols, and Coste *et al.* (18), studying locusts fed plant diets containing high levels of cyclopropyl sterols. The possibility that one or more metabolites were toxic to the insects was ruled out by a careful examination of the sterol composition of the larvae; only the test compound or a utilizable sterol metabolite was detected in the insect.

A second possibility is that inadequate absorption of sterols from the intestinal gut led to a sterol content in the larvae that was below the threshold value necessary to promote growth, and therefore generated a nonutilizable sterol. This line of reasoning might imply that the level of sterol, and not small changes in the structure, gives rise to nonutilization. However, as we subsequently discovered, both the amount and type of sterol absorbed by the insects determine the sterol efficacy. All the nonutilizable sterols were recovered from larvae in the range between 0.6 and 6 μg per insect. Larvae treated with a nonutilizable sterol contained trace amounts of cholesterol that ranged between 80 and 300 ng of cholesterol. The larval cholesterol was most likely derived from the agar and/or from carryover from cholesterol originally present in the eggs (*ca.* 80 ng of cholesterol per egg was detected, similar to the cholesterol content of eggs from the fire ant, Ref. 15). Since the treatment with 24-methylcholesta-5,23-dien-3β-ol sparred cholesterol to grow and mature, the lower limit of cholesterol for development appears to be at or above 300 ng per insect. Although more study is warranted, we hypothesize that about 18 μg of sterol is required for normal *H. zea* growth (based on sparing studies with cholesterol and 24-dihydrolanosterol); of that utilizable sterol, approximately 10.0% is required for ecdysteroid production (based on the level of cholesterol generated in the 24-methylcholesta-5,23-dien-3β-ol treatment), the remaining sterol functioning as a membrane insert.

Sterols that may be grouped into the utilizable category contain a 3β-hydroxyl group, a planar nucleus with a Δ^5-bond, and an intact side chain of 8 to 10 carbon atoms. Neither the geometry of the 24(28)-ethylidene group nor the stereochemistry of the C-24 alkyl group was essential for activity, since isofucosterol and fucosterol, and sitosterol and 24-episitosterol (clionasterol) were found to support growth, and each compound was converted to cholesterol in high yield. A series of cholesterol analogs with side-chain modifications at C-24 and double bonds introduced in the side chain at Δ^{23}-, Δ^{24}-, and Δ^{25}-positions induced pronounced growth-retarding effects, suggesting an important role in the structure of the side chain of sterols grouped into the nonutilizable category. Trace amounts of cholesterol in the clereosterol treatment suggest that *H. zea* may not synthesize a $\Delta^{25(27)}$-reductase enzyme. Alternatively, since desmosterol (cholesta-5,24-dien-3β-ol) and stigmasterol may be considered intermediates to cholesterol, then *H. zea* must possess at least two side-chain reductase-type enzymes, viz., a $\Delta^{24,25}$-reductase and a $\Delta^{22,23}$-reductase. Based on our studies with stigmasterol and ergosterol and that of Ritter and Nes, who also examined ergosterol and brassicasterol (24β-methylcholesta-5,22*E*-dien-3β-ol), we infer that catalytic competence of the $\Delta^{22,23}$-reductase is sensitive to the size of the 24-alkyl group, since the 24-methylsterols were converted to a saturated side chain by *H. zea* less effectively than 24-ethylsterols. 24-Methyldesmosterol and 24-ethyldesmosterol were found to be recovered from the insects in unchanged form (Table 1), suggesting that the 24,25-reductase is also sensitive to the addition of substitutents which can can add steric bulk at position C-24. From the $\Delta^{23(24)}$-24-alkyl treated larvae, trace cholesterol was recovered. This low level of cholesterol is assumed to be from the diet rather than from metabolism of the dietary sterol. Thus, the insect may also not synthesize a $\Delta^{23(24)}$-reductase enzyme. The inability for either the $\Delta^{23(24)-}$ or $\Delta^{25(27)}$-sterols to satisfy the sterol requirement of *H. zea* suggests that there is a high degree of regiospecificity for the double bond to be located at C-24(28) in the side chain for C-24 dealkylation pathway to proceed to desmosterol production.

The corn earworm can tolerate certain modifications in the nucleus, as shown earlier by Ritter (8–10), studying a series of Δ^0-, Δ^5-, and Δ^7-, and Δ^8-sterols. However, we found that many phytosterols that are transformed naturally by plants, such as those with a 4,4-dimethyl group (24-dihydrolanosterol, lanosterol, and cycloartenol), a 9β,19-cyclopropane

group (24-dehydropollinastanol and cycloartenol), a $\Delta^{8(9)}$-bond (cholest-8(9)-en-3β-ol) were ineffective nutritional supplements and were recovered from larvae in unchanged form (Table 1), suggesting a much stricter sterol specificity than was recognized by Ritter (8–10). For mechanistic reasons, 24-dihydrolanosterol should pass through cholest-8(9)-en-3β-ol onto cholesterol (19). Our inability to detect (trap) cholest-8-en-3β-ol in the 24-dihydrolanosterol treated larvae is consistent with our observation that 24-dihydrolanosterol was not converted to cholesterol and that the cholesterol in the larvae was from contamination (carryover from the eggs and agar). The accumulation of significant amounts of cholest-5,7-dien-3β-ol (7-dehydrocholesterol) from the ergosterol-treated insects suggests there is little metabolism of $\Delta^{5,7}$-sterols by the insects, which is consistent with the findings of Ritter (9). We interpret these results to imply that *H. zea* fails to synthesize several enzyme systems synthesized by plants that act on the nucleus of sterols: a 4-demethylase, a cyclopropyl to Δ^{8}-isomerase, a $\Delta^{8(9)}$-to $\Delta^{7(8)}$-isomerase, a Δ^{7}-reductase, and a Δ^{7}-desaturase. The inability of *H. zea* to metabolize either lanosterol or cycloartenol is in sharp contrast to the strong 24,25-reduction of these two sterols by the tobacco hornworm (4,11). Ritter (9) claimed that *H. zea* could metabolize lanosterol to cholesterol, suggesting that *H. zea* synthesized all the requisite enzymes to carry out lanosterol turnover to a Δ^{5}-sterol. However, our results, reported in Table 1, do not support her findings.

In postulating a structural function for nonutilizable sterols, we have implied that the sterols are incorporated *per se*, or at any rate are not converted into any metabolically useful products. Support for this view has been obtained by feeding *H. zea* larvae, for instance, 24-methylcholesta-5,23-dien-3β-ol, and by showing that the bulk of it can be recovered in unchanged form. Alternatively, utilizable sterols are those compounds that may function in both roles, nonmetabolic and metabolic. It is concluded that some nonutilizable sterols can spare (e.g., 24-methyldesmosterol), but cannot replace, cholesterol in the nutrition of *H. zea* and these sterols serve in some structural rather than metabolic function. Either enzyme specificity acts on the nonutilizable sterols, such that one or more substituents on the molecule interfere with normal transformation of the phytosterol to ecdysteroid, or the insect lacks one or more enzyme systems necessary to convert the compound to cholesterol. In the case of 24-methylcholesta-5,23-dien-3β-ol, the double bond is positioned incorrectly for 24(28)-epoxidation during the initial phase of C-24-dealkylation, whereas for 24-dehydropollinstanol the insect lacks minimally an active 9β,19-cyclopropyl to $\Delta^{8(9)}$-isomerase. The inability to transform clereosterol, with a $\Delta^{25(27)}$-bond, to cholesterol is consistent with the mechanism of C-24 dealkylation which requires hydrogen migration from C-25 to C-24 during 24,25-double bond formation (2, 4). It is expected that plants that can be treated with specific sterol biosynthesis inhibitors or genetically engineered to produce sterol enzymes that give rise to altered enzyme efficiencies and product distributions will possess modified phytosterol compositions that accumulate 4,4-desmethyl 9β,19-cyclopropyl sterols or may generate clereosterol and related novel $\Delta^{23(24)}$-and $^{\Delta 24(25)}$-24-alkyl sterols as replacements of the normal Δ^{5}-24-alkyl sterols. This potentially environmentally friendly biotechnology can lead to broad-based insect resistance.

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Remote Functionalization of the Steroid Side-Chain

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ABSTRACT: By using classical methods of organic synthesis, the introduction of chemical modifications into the saturated side-chains of steroids usually requires a multistep synthesis to construct new side-chains to be added to the steroid nucleus. In order to circumvent these earlier methods, new procedures have been developed to directly introduce functionality onto the steroid side-chain to produce useful products. These initial products may also provide an entry toward the further modification of the side-chain to produce steroids which could previously be obtained only with great difficulty.

Lipids 32, 1325–1330 (1997).

The side-chains of naturally occurring steroids vary in their structure and composition (1–4). Individually, they represent unique challenges to the organic chemist with the task of chemically modifying intact side-chains or constructing new ones through multistep synthesis. Chemical modification of an existing side-chain, which may contain unsaturation, is a much easier task than the construction of a new side-chain which may be time-consuming and may require many chemical steps. Many naturally occurring steroids contain side-chains that are saturated and are devoid of any functionality which the chemist might use as an intermediate in the modification process.

Chemical functionalization of the saturated side-chain of steroids (e.g., cholesterol) is a difficult problem for the organic chemist. The need to modify the saturated side-chain of certain steroids may arise through the study of enzymatic transformations of the side-chain and the requirement to have authentic standards for the identification of potential metabolites. Also, the synthesis of biochemical inhibitors would require introducing functionality at selected positions on the side-chain. Collectively, the necessity to modify the saturated side-chain of steroids has been the driving force for developing facile methods to accomplish this goal.

Historically, the side-chain of steroids could be modified by using unsaturation already available in a naturally occurring steroid. In this way, steroids such as desmosterol (cholesta-5,24-dien-3β-ol) (5,6), fucosterol (24-methylene-cholest-5-en-3β-ol (7,8), stigmasterol (24α-ethylcholest-5,22-dien-3β-ol) (9,10), and lanosterol (lanosta-8,24-en-3β-ol) (11–14) easily could be converted into side-chain derivatives for various purposes. Unfortunately, steroids such as desmosterol and fucosterol may not be readily available in larger quantities and are costly starting materials.

The earlier literature, describing the construction of side-chains from an existing steroid nucleus, has been reviewed (15). There are also many more recent examples of these types of modification reactions, and we have attempted to categorize a number of these which may be of interest. One approach is to use a C-17 ketone (usually 3β-hydroxyandrost-5-en-17-one) as a starting material and convert it into a side-chain-bearing steroid in a multistep synthesis (16–18). Another approach involves the use of a C-20 ketone (19–22) or aldehyde (23) as a starting material, which is modified into an appropriate side-chain derivative. Also, an aldehyde (24–27), alcohol (28), or carboxylic acid (29) function at C-21 has been used to lengthen the side-chain of steroids. Modification of a C-22 alcohol (30) or carboxylic acid (usually 3β-hydroxy-5-cholen-22-oic acid) (31) has been used to extend the terminal carbons of side-chains. In a similar manner, C-24 aldehydes and alcohols (31) (usually derivatives of 3β-hydroxy-5-cholen-24-oic acid) have also been used to modify and extend the existing side-chains of steroids.

An alternative approach is to directly introduce side-chains, with various functional groups, onto an existing steroid nucleus. This approach usually requires a multistep synthesis, and in most cases the final product is produced in low or very moderate overall yields. This area of research continues to be very active, and many different investigators have directed their talents to the construction of steroid side-chains. These may contain diverse functionality, including unsaturation, which could be further modified in an additional sequence of reactions. This approach usually involves constructing a side-chain by modifying carbon atoms 17–24 (15–33).

An alternative method to the functionalization of steroid side-chains involves the direct introduction of a modification into the side-chain in a single chemical reaction. This approach is termed "remote functionalization" and represents a rapidly developing area in steroid chemistry which promises to streamline the synthesis of many difficult-to-obtain steroids. Through the earlier work of Breslow and colleagues (34–38), methods for the remote functionalization of the steroid nucleus have become well-known reactions. The functionalization of remote positions on the steroid nucleus and side-chain represents some of the most important advances in

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the steroid field. Remote functionalization of a saturated steroid side-chain usually involves oxidation on the tertiary carbon at C-25 to produce a 25-hydroxysterol. In most studies, these reactions have been developed in the cholesterol series, and the end product of these reactions is cholest-5-en-3β-diol (25-hydroxycholesterol).

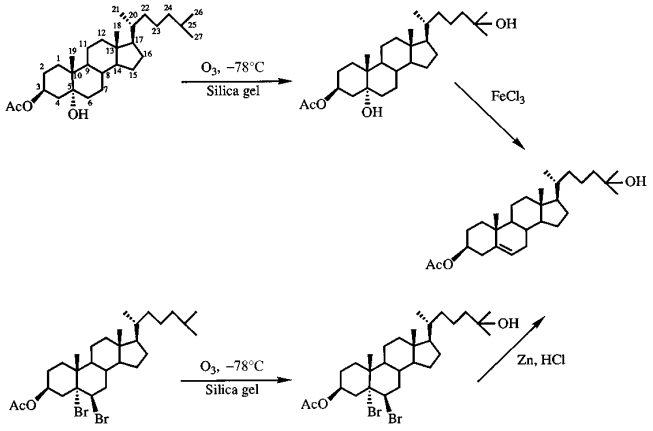
The predominant target molecule in many studies has been 25–hydroxycholesterol owing to its interesting biological properties and its ability to be dehydrated to desmosterol which can be further chemically modified. 25-Hydroxycholesterol is a member of a class of sterols known as oxysterols which possess a second oxygen function in addition to that at C-3 and are known to have diverse biological activities (39–48). Some of these include cytotoxicity, atherogenicity, carcinogenicity, mutagenicity, hypocholesterolemia, and various effects on specific enzymes. 25-Hydroxycholesterol is known to be a very potent inhibitor of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, a key regulatory enzyme in cholesterol biosynthesis ($IC_{50} < 1$ μM) (39,42).

Repression of the synthesis of immunoprecipitable HMG-CoA reductase by this oxysterol has been demonstrated by Faust *et al.* (49) in a Chinese hamster ovary (CHO)-derived compactin-resistant cell (UT-1) line that overproduces the enzyme and by Sinensky *et al.* (50) in CHO cells. Lutsky *et al.* (51) have shown that the level of mRNA for the reductase in UT-1 cells is reduced in the presence of oxysterol. Similar results were obtained by Kandutsch *et al.* (52–54) in wild-type CHO cells. These results were utilized by Kandutsch and Thompson (55), who proposed the following model for the regulation of HMG-CoA reductase activity by oxysterols: The oxysterol binds to a cellular protein, and the resultant oxysterol–protein complex then acts to repress transcription of the gene for reductase production. In support of this model, Kandutsch *et al.* (52–54) have isolated a protein which exhibits high affinity and low capacity for 25-hydroxycholesterol and other oxysterols which are known to suppress reductase production. In support of this model, Kandutsch *et al.* (54,56–58) have isolated a protein which exhibits high affinity and low capacity for 25-hydroxy and other oxysterols which are known to suppress reductase activity. Although 25-hydroxycholesterol has been isolated from cells, there is no direct evidence that this oxysterol functions as a natural regulator of cholesterol biosynthesis (59). This model was in direct contrast to the prevailing view that cholesterol, accumulating in cells after endocytosis of low density lipoprotein (containing cholesteryl esters), is the regulatory messenger molecule (60).

Primary metabolism in mammalian systems is known to produce side-chain oxysterols. Derivatives of cholesterol hydroxylated in the 25- or 26-positions are produced in liver during bile acid synthesis. Another mode of oxysterol biosynthesis has been described which utilizes the isopentenoid pathway to produce side-chain derivatives of cholesterol and lanosterol (61). Such compounds are derived from squalene 2,3-epoxide by the introduction of a second oxygen function to form 2,3;22,23-dioxidosqualene prior to cyclization. Thus, this intermediate has been shown to form 24(*S*),25-epoxylanosterol, 24(*S*),25-epoxycholesterol, and 25-hydroxycholesterol in mammalian systems (62–65). 24(*S*),25-Epoxycholesterol has been isolated from cultured mouse L-cell, Chinese hamster lung fibroblasts, and human liver (59). These oxygenated side-chain derivatives have been shown to be potent inhibitors of HMG-CoA reductase and sterol biosynthesis and possess a high affinity for the oxysterol binding protein (59,62–65). These results add further support to the hypothesis that oxysterols may be natural regulators of cholesterol biosynthesis in mammalian cells (53,65,66). This dioxidosqualene pathway has recently been reviewed (65,66). Also, its occurrence in plants, animals, and microorganisms (67,68) and its evolution in a variety of organisms have been reviewed (69).

Early attempts to obtain 25-hydroxycholesterol directly from cholesterol included attempts to enhance the air or autoxidation process (46). Fieser *et al.* (70) examined cholesterol samples differing only in age and concluded that cholesterol undergoes oxidation to the 3β,25-diol on storage in the crystalline state in the presence of air. Bubbling air through a refluxing solution of crystalline cholesterol in benzene only resulted in air oxidation products other than the 3β,25-diol. It was therefore concluded that the observed selectivity of hydroxylation at the C-25 position, in the air oxidation of cholesterol, was a consequence of the regular arrangement of the steroid molecules on the major faces of the plate-like crystals (71). On this basis a more general hypothesis was advanced that when molecules are observed in a close-packed array the mutual steric effect of one upon the other may hinder reactions at the usual position and thus enhance oxidation of more exposed terminal areas. Using this approach, experiments were conducted in which barium sulfate or neutral alumina bearing absorbed cholesterol was heated in air or oxygen in the presence of an initiator, or irradiated with ultraviolet light. In each of these experiments some 3β,25-diol was formed, but the yields (approximately 3%) were less than those obtained by autoxidation of the crystalline steroid, and the product mixtures were very complex (72). The poor results of these earlier experiments are not surprising since the C-5 double bond, which is a major site for autoxidation, is not chemically protected and thus contributes to the low yields of the 3β,25-diol.

A related approach to the direct functionalization of side-chains with diatomic oxygen is the use of ozone. Mazur and colleagues (73,74) have developed useful procedures for the ozonolysis of steroid substrates adsorbed on dry silica gel to introduce oxygen into unactivated tertiary C-H bonds. This method involves preadsorption of substrate on chromatographic-grade silica gel and passage over it of ozone at temperatures between −75 and −45°C, followed by elution with an appropriate organic solvent. The reactivity of the tertiary C-H bonds toward ozone depends both on the electronegativity and the steric availability of the carbon atom (73). In this way, the dry ozonation of 1α,3β-diacetoxy-6,7-dibromo-5α-cholestane led to the C-25 hydroxylated derivative as the only

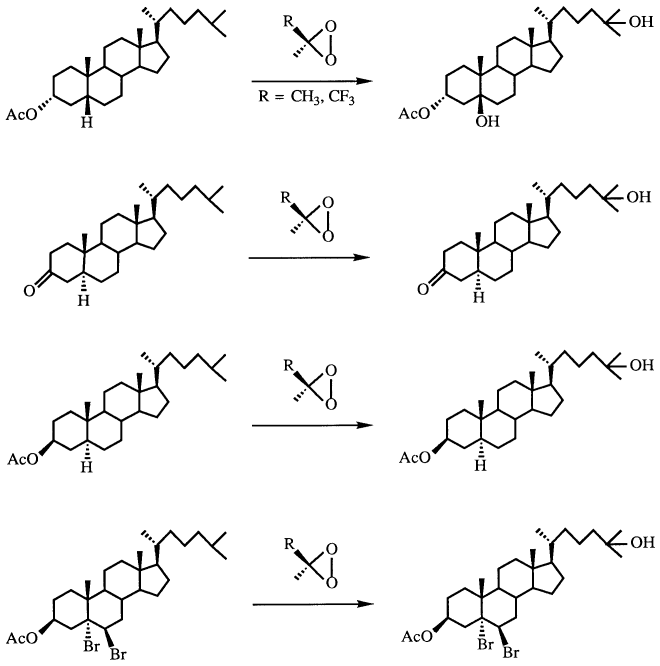


SCHEME 1

isolated product (11% conversion and 51% yield) (73). In a later study, a number of saturated cholestane derivative substituted at positions 5, 6, and 7 which served as protecting groups for ring-B double bonds were ozonized on silica gel to produce the C-25 hydroxylated products (Scheme 1). Also, a 3β-acetoxy-6,7-dibromo-5α-cholestane was ozonated to its C-25 hydroxy derivative and further dehydrobromination led to the respective 5,7-diene (73). The protecting groups, for the ring-B double bonds, served a dual function. In addition to their protecting role, they sterically hindered the approach of ozone to the other tertiary carbon atoms on the steroid nucleus and allowed an increased yield of the C-25 hydroxylated products.

In other studies of side-chain remote functionalization, the Gif system has been used to oxidize cholestane derivatives to the corresponding C-20 ketones (75–78). The oxidation of cholest-4-en-3-one by the Gif system to give progesterone has been studied. The optimal temperature was ~20°C, and a reaction temperature below 0°C gave 25-hydroxycholest-4-en-3-one as the major product (79). A long-range intramolecular functionalization of an irradiated 7α-hypoiodite derivative of cholestane has been reported to yield the C-25 hydroxysteroid as a final product (80) . Another report describes the C-25 hydroxylation of cholesterol by a membrane-spanning Mn(III) porphyrin positioned in a synthetic bilayer assembly (81). This synthetic porphyrin is capable of mimicking the hydroxylation activity of certain cytochrome P-450 enzymes.

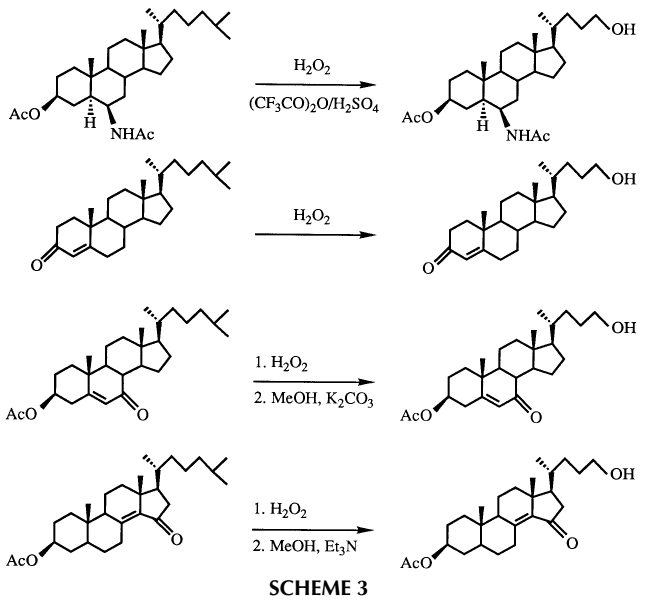
Recently, remote functionalization to produce hydroxylation at C-25 has been achieved using dioxiranes. Dioxiranes had previously been described as highly effective reagents in oxyfunctionalization of saturated hydrocarbons and the steroid nucleus (82,83). Both dimethyldioxiran and methyltrifluoromethyl dioxirane converted 3α-acetoxy-5β-cholestane to 3α-acetoxycholest-5β,25-diol in a simultaneous double oxyfunctionalization (Scheme 2) (84). In a related study, the direct and high yield oxyfunctionalization of 5α-cholestan-3-one, 3β-acetoxy-5α-cholestane, and 3β-acetoxy-5α,6β-dibromocholestane was achieved to produce the C-25 hydroxy derivative under mild conditions using dimethyldioxirane or its trifluoromethyl analog (Scheme 2) (85). The



SCHEME 2

latter steroid was cleanly (yield 93%) converted to 3β-acetoxycholest-5-en-25-ol upon debromination with zinc in acetic acid, thus restoring the $\Delta^{5,6}$ double bond that had been protected by the dibromide.

Another successful approach to remote functionalization involves the use of a mixture of hydrogen peroxide and trifluoroacetic anhydride (trifluoroperacetic acid) together with sulfuric acid to oxidize and cleave steroid side-chains. Deno *et al.* (86,87) initially developed this method which was also utilized by Takano and Ogasawara (88) to oxidize selected steroids to the corresponding C-24 alcohols. In this way, an amide derivative of cholesterol (which was finally converted



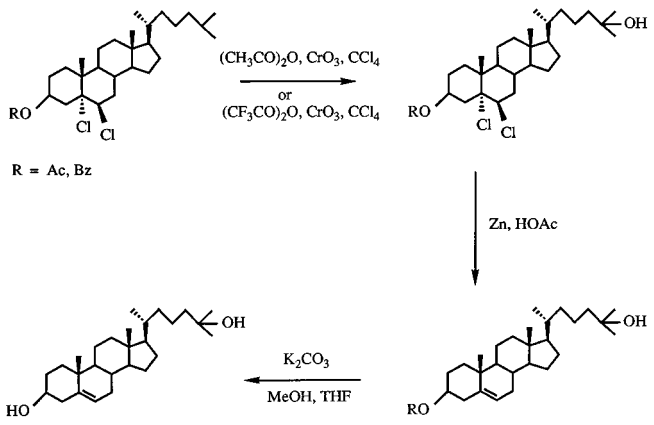
SCHEME 3

into the $\Delta^{5,6}$ double bond), cholest-4-en-3-one, and 3β-acetoxycholest-5-en-7-one were oxidized to give the C-24 hydroxy derivatives in modest yields (17–19%) (Scheme 3). A significant improvement in this procedure was described by Schroepfer *et al.* (89–92) which allowed the isolation of the end product in much higher yields. In this procedure, 3β-acetoxycholest-8(14)-en-15-one was oxidized to a crude mixture of products which was further treated with triethylamine in methanol to provide 3β-acetoxy-24-hydroxy-chol-8(14)-en-15-one in 61% yield (Scheme 3). In these studies, Schroepfer *et al.* described the use of their C-24 alcohol as a key intermediate to modify the steroid side-chain.

An additional approach to remote functionalization that has been useful for introducing a 14α-hydroxyl group onto steroids devoid of a side-chain is the use of chromyl acetate (93–95). This reagent is made *in situ* from acetic anhydride and chromium trioxide and was previously used for the oxidation of hydrocarbons (96). We became interested in this reagent for possible development into a new method for direct hydroxylation at C-25. This procedure possessed the desirable properties of being simple, convenient, and applicable to larger-scale reactions. After optimization, we were able to isolate the C-25 hydroxy derivative of 3β-acetoxy- or 3β-benzoyloxy-5α,6β-dichloro-5α-cholestane in yields of 14–18% (Scheme 4) (97,98). The success of this procedure depends on maintaining some starting material during the course of reaction to prevent further oxidation of the initially formed C-25 hydroxylated product. The accessibility of the relatively less hindered C-25 on the side-chain allowed an initial selective oxidation at this position followed by partial oxidation of other tertiary carbons on the steroid nucleus which would result in complex oxidation products. The initial C-25 hydroxylated product was then treated with zinc in acetic acid to remove the 5α,6β-dichloride and restore the $\Delta^{5,6}$ double bond (94), and the ester function at C-3 was removed by mild base hydrolysis (12) to produce 25-hydroxycholesterol (Scheme 4).

In related experiments, we were able to improve these results through the use of the reagent trifluorochromyl acetate which can be prepared *in situ* from the reaction of trifluoroacetic anhydride and chromium trioxide (99). Again, this reagent system has a history of oxidizing hydrocarbons (99). After optimization and using the same techniques described, we were able to isolate the C-25 alcohol of the previously described starting material in yields of 40–60% (Scheme 3) (97,98). We believe this procedure will prove to be a useful method for the remote functionalization at C-25 in steroid side-chains and will be an attractive alternative to other known procedures.

In conclusion, the remote functionalization of steroid side-chains is a continuing challenge in synthetic organic chemistry. The application of new and known reagents, with a demonstrated ability to oxidize hydrocarbons, has the potential to increase the number of reagents which can be used in the steroid fields for this purpose.



SCHEME 4

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Studies on *N*-Nitroso Bile Acid Amides in Relation to Their Possible Role in Gastrointestinal Cancer

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ABSTRACT: Cancers of the gastrointestinal tract account for a large proportion of neoplastic diseases which afflict humans. The etiology of gastrointestinal cancer has been attributed in part to exogenous carcinogens, such as food substances and environmental pollutants. Recent hypotheses suggest that carcinogens may arise endogenously. Evidence suggests that some bile acids and their isomeric metabolites may be involved in the pathogenesis of colon cancer. However, the mechanism responsible for their cancer-promoting effect is not clear. We and others propose that one mechanism for the mitogenic effects of bile acids may be *N*-nitrosation of their glycine and taurine amides; human gastric aspirates do contain small quantities of *N*-nitroso compounds of other substrates. Many foods contain nitrites and nitrates, which can react with bile acid amides to form *N*-nitroso derivatives. Our recent studies demonstrated the potential for *N*-nitroso conjugate formation from ursodeoxycholic acid, a 7β-epimer of chenodeoxycholic acid used as a drug Actigall® to dissolve gallstones. The *N*-nitroso derivative of this compound, a direct-acting carcinogen, has a long half-life and, once nitrosated, is stable enough to survive passage through the gastrointestinal tract. We describe the synthesis of *N*-nitrosated derivatives of various bile acid conjugates and mechanisms of decomposition of (*Z*)- and (*E*)-bile acid diazoates. Studies of the effects of enzymes such as cholylglycine hydrolase on the *N*-nitroso bile acid conjugates and their reaction with DNA are also described. These studies may have important implications in the interplay of diet with endogenous substrates in the etiology of cancers of the stomach, liver, and colon.

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Gastrointestinal cancer is a major malignancy worldwide (1,2). In 1995, the American Cancer Society reported 124,000 deaths in the United States from gastrointestinal cancers, second only to the 157,000 deaths due to lung cancer (3). Epidemiologic evidence supports the theory that dietary fiber consumption is protective against gastric and colorectal cancer (4) especially when the fiber is derived from a diet high in fruits and vegetables. It is difficult to separate the epidemiological effects of high fiber from the low fat content of such diets.

A low-fat diet may protect against colon cancer. Data from the Harvard School of Public Health (5) indicated that men with the lowest fat intake—an average of 24% of calories from fat—had only half the rate of colonic adenomatous polyps, a precursor of colon cancer, as men with the highest fat intake of 41%. In countries like Japan, where the average diet contains less than 10% of calories from fat, there is less colon (and breast) cancer.

The mechanism for the increased cancer rate in individuals and groups ingesting a high-fat diet is not clear. Recent interest has focused on the carcinogenic role of *N*-nitroso compounds derived from the consumption of preserved, smoked, and cured foods (6–9). A variety of *N*-nitroso compounds have been found to produce tumors in more than 40 animal species (6). In addition to *N*-nitroso compounds derived from exogenous carcinogens, such as food substances and environmental pollutants, endogenous sources have been identified. Thus, gastric cancer has been associated with chronic infection with *Helicobacter pylori*; other cancers are associated with chronic bacterial, viral, and parasitic infections (10–12). Nitric oxide, a short-lived free radical produced by many cells for important physiological functions, is increased in these infections and can produce endogenous carcinogenic *N*-nitroso compounds (13). Recent data have suggested that enhanced production of NO has the potential to induce mutagenic and possibly carcinogenic alterations in the gut epithelium *via* the formation of potent *N*-nitrosating agents derived from the spontaneous decomposition of NO in oxygenated solutions (13,14). In this regard, we and others have suggested that some bile acids, synthesized in the liver from cholesterol for the purpose of aiding in the digestion of fats, may be *N*-nitrosated in the stomach or intestines to form potentially mitogenic compounds (15,16). The most likely human exposure to direct-acting *N*-nitroso compounds (*N*-nitroso bile acid amides) would result from their *in vivo* formation because of their inherently unstable chemical nature.

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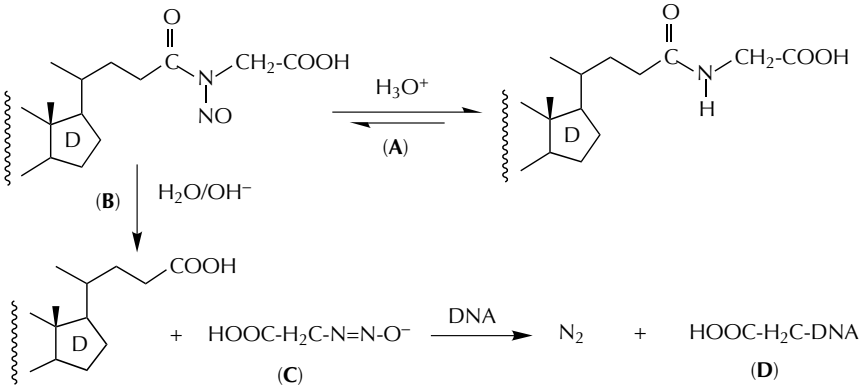
Abbreviations: CA, cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid); CDCA, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid); DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid); EEDQ, *N*-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline; ESI, electrospray ionization; Et_3N, triethylamine; FAB, fast atom bombardment; GCA, glycocholic acid; GUDCA, glycoursodeoxycholic acid; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOGC, *N*-nitrosoglycocholic acid; NOGDCA, *N*-nitrosoglycodeoxycholic acid; NOGUCA, *N*-nitrosoglycoursocholic acid; NOGUDCA, *N*-nitrosoglycoursodeoxycholic acid; NOTC, *N*-nitrosotaurocholic acid; NOTUDCA, *N*-nitrosotauroursodeoxycholic acid; TLC, thin-layer chromatography; TUDCA, tauroursodeoxycholic acid; UCA, ursocholic acid (3α,7β,12α-trihydroxy-5β-cholan-24-oic acid); UDCA, ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid).

Considerable research has been directed toward formation and biological activation of the *N*-nitrosamines; less effort has focused upon the *N*-nitrosamides, which require no activation and are known to be potent direct-acting mutagens and carcinogens (17–19). As described above, the major source of nitrosable amides in the human body is the conjugated bile acids. Hepatocytes synthesize bile acids as oxidative degradation products of cholesterol (20,21). Then, the bile acid-conjugating enzyme, bile acid coenzyme A: amino acid: *N*-acyltransferase, specifically couples the bile acids with either of two amino acids, taurine or glycine (22). The conjugated bile acids enter the upper intestinal tract and, having performed their function of assisting in the absorption of fat, they are reabsorbed and recirculated in the enterohepatic circulation (23). The "pool" of bile acids circulates two or three times during each meal, or five to ten times daily. Since the body maintains a constant amount of 2 to 4 g of bile acids, theoretically 10 to 40 g of conjugated bile acids travel through the small intestine daily (20–23). At the terminal ileum, special receptors actively absorb most of the bile acids and return them to the liver in portal venous blood. The remaining bile acids enter the large intestine, where they are degraded by intestinal bacteria, and free bile acids are passively absorbed in the colon. However, a percentage of the bile acids, approximately 500 mg or more per day, are not reabsorbed by the terminal ileum or colon. Consequently, some are excreted in the feces whereas a few may bind to the tissues in the region of the ileum and colon (21–24).

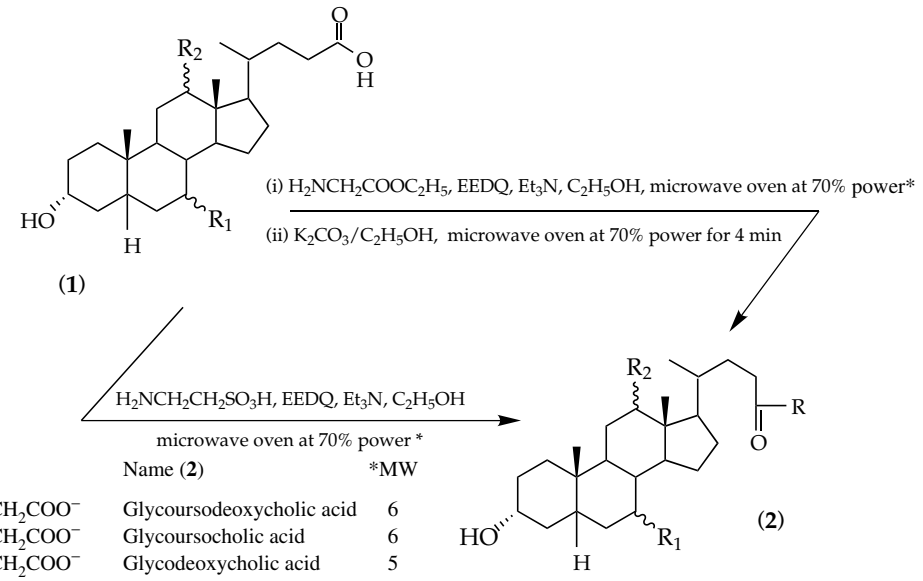
The most likely human exposure to direct-acting *N*-nitroso bile acid conjugates would result from their *in vivo* formation potentially at several organ sites. A number of investigators have proposed that nitrosamides are produced in the stomach from amides and nitrite derived from food and that they act in that organ to induce cancer (25–30). The acidic environment of the stomach after meals is ideal because amide nitrosation is catalyzed by acid (28). Nitrite enters the stomach (*ca*. 4.3 mg/person/day) from two sources: 20% arises from nitrite in the diet, and 80% from the reduction of salivary nitrate by oral and gastric bacteria, especially under mildly acidic conditions (28). Cured meat, baked goods, cereals, and vegetables contribute to dietary nitrite, thus increasing gastric nitrite levels at the same time that the conjugated bile acids are circulating. Furthermore, nitrite disappears from the stomach because gastric acid converts it to nitrous acid (HNO_2), which upon decomposition to NO reacts with nitrogen compounds such as the nitrosamides to yield *N*-nitroso derivatives (29). Nitrous acid was the reagent used in the chemical synthesis of *N*-nitroso bile acid conjugates in the present study (15). Nitrosation can also be mediated by bacteria and activated macrophages in infected and inflamed organs (30). Endogenously occurring *N*-nitroso compounds may induce DNA modification by the formation of DNA adducts. In addition, time–dose relationships observed for tumor formation in animals indicate that lifetime or chronic exposure to even low levels of certain carcinogenic *N*-nitroso compounds may represent a greater cancer risk to people because of their long life spans (31).

Thus far, studies of *N*-nitrosated bile acid conjugates have been limited to derivatives of a naturally occurring hydrophobic bile acid, cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid, CA); *N*-nitrosotaurocholic acid (NOTC) and *N*-nitrosoglycocholic acid (NOGC) (31,32) were found to be mutagenic, in the absence of activation, in both forward and reversion bacterial mutation assays, while their precursors taurocholic acid and glycocholic acid were not. In a human lymphoblast assay, NOGC was approximately 9000 times more potent than NOTC in causing mutations (31). Investigation of the carcinogenic activities of NOTC and NOGC in rats showed that both induced significant levels of hepatocellular carcinoma and malignant stomach tumors (32).

Furthermore, the most significant studies concerning the formation and fate of nucleic acid and protein adducts derived from *N*-nitroso glyco bile acid conjugates (NOGC) have been reported from the laboratory of Shuker *et al*. (33). ^{14}C-NOGC reacted with calf thymus DNA to give a number of carboxymethylated adducts, namely, 7-carboxymethylguanine (0.025%), 3-carboxymethyladenine (0.006%), and O^6-carboxymethylguanine (0.0026%) (33,34). The behavior of NOGC in forming carboxymethyl electrophilic alkylating agents which bind with DNA nucleotides is consistent with its expected decomposition as an *N*-nitrosamide (see Scheme 1, a generalized description of the decomposition of *N*-nitroso glyco-bile acid conjugates) (15,16,18,19,26,29,30). These results suggested that

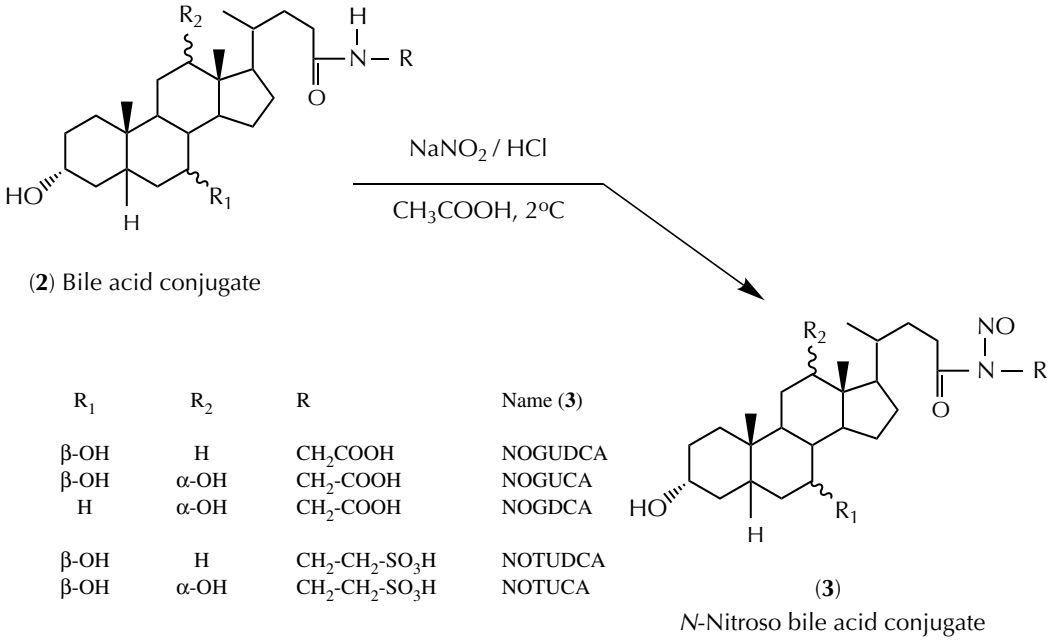


SCHEME 1



	R_1	R_2	R	Name (**2**)	*MW
a	β-OH	H	$-HNCH_2COO^-$	Glycoursodeoxycholic acid	6
b	β-OH	α-OH	$-HNCH_2COO^-$	Glycoursocholic acid	6
c	H	α-OH	$-HNCH_2COO^-$	Glycodeoxycholic acid	5
d	β-OH	H	$-HNCH_2CH_2SO_3^-$	Tauroursodeoxycholic acid	5
e	β-OH	α-OH	$-HNCH_2CH_2SO_3^-$	Tauroursocholic acid	5

SCHEME 2



	R_1	R_2	R	Name (**3**)
a	β-OH	H	CH_2COOH	NOGUDCA
b	β-OH	α-OH	CH_2-COOH	NOGUCA
c	H	α-OH	CH_2-COOH	NOGDCA
d	β-OH	H	CH_2-CH_2-SO_3H	NOTUDCA
e	β-OH	α-OH	CH_2-CH_2-SO_3H	NOTUCA

SCHEME 3

O^6-methylguanine, which has been observed in DNA from gastrointestinal tissues, may be derived from intragastric nitrosation of glycine or related compounds (34).

Our laboratory's long-term interest in the chemistry, biochemistry, and physiology of naturally occurring hydrophobic and hydrophilic bile salts has encouraged us to investigate in detail the role of the *N*-nitrosated derivatives of bile acid conjugates in the development of cancer, particularly those of the 7β configuration. Chemical synthesis, structural analysis, and decomposition of *N*-nitroso amides of some hydrophilic bile salts (see Scheme 2, on conjugates of bile acids with glycine and taurine, and Scheme 3, on *N*-nitrosation of bile acid conjugates) were recently reported from our laboratory (15), along with their pH-dependent activities and stabilities, the half-life ($t_{1/2}$), and stability under conditions likely to be encountered in the gastrointestinal tract (15).

The present studies summarize the chemistry and biochemistry of various *N*-nitroso derivatives of bile acids and their characterization by high-resolution ^{1}H nuclear magnetic resonance (NMR) and fast atom bombardment–mass spectrometry (FAB–MS) or electrospray ionization–MS (ESI–MS). Additional studies such as mechanisms of decomposition of (*Z*)- and (*E*)-bile acid diazoates and the effects of enzymes such as cholylglycine hydrolase on the *N*-nitroso bile acid conjugates and their mutagenic potential by reacting with DNA are also described.

EXPERIMENTAL PROCEDURES AND RESULTS

Ursodeoxycholic acid (UDCA), ursocholic acid (UCA), and deoxycholic acid (DCA) of pharmaceutical grade were donations of Prodotti Chimici e Alimentari S.p.A. (Alessandria, Italy). Reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated Silica Gel-60 plates (F-254; Merck). Solvent systems 26:8:4:2 $CHCl_3$/MeOH/CH_3COOH/H_2O (A), 40:6:4 $CHCl_3$/MeOH/CH_3COOH (B), 70:50:5 (C), $CHCl_3$/$(Me)_2CO$/MeOH and 70:50:10 $CHCl_3$/$(Me)_2CO$/MeOH (D) were used as appropriate (35,36). The spots were made visible with 3.5% phosphomolybdic acid in isopropanol and 10% (wt/vol) sulfuric acid. A commercial microwave oven (Whirlpool, 2450 MHz, total cooking power 650 Watts) was used in irradiation experiments. The reaction mixture was held in an Erlenmeyer flask, covered with an inverted funnel, and placed in a beaker containing water (*ca.* 150 mL). The beaker served as a heat sink to dissipate excess microwave energy (37–39).

The ^{1}H NMR spectra were recorded on a Varian XL-400 (400 MHz) spectrometer in dimethylsulfoxide (DMSO) solution. Proton chemical shifts are reported relative to tetramethylsilane. FAB–MS studies were conducted in the positive ion mode using nitrobenzyl alcohol as a matrix, dimethylsulfoxide as the solvent, and NaCl to enhance the peaks (35,36). ESI–MS was performed in the negative ion mode in MeOH as the solvent as described previously (15).

The bile acid conjugates were prepared using a new technique (37–39) which employs a domestic microwave oven, as shown in Scheme 2 (15,40,41). This new methodology is currently under active use in our laboratory to synthesize substrates on a large scale for a variety of important clinical research projects.

Glyco-bile acid conjugates **2a**–**2c** (Scheme 2) were synthesized by reacting the corresponding free bile acid **1** with the ethyl ester of glycine in the microwave, followed by hydrolysis of the ester with potassium carbonate and ethanol, also in the microwave oven. Tauro-bile acid conjugates **2d** and **2e** were synthesized by the reaction of **1** with taurine in the microwave oven. After the usual work-up, the desired products were isolated in good to excellent yields (15). A representative procedure is provided next.

*Glycoursodeoxycholic acid (GUDCA) (***2a***, Scheme 2).* UDCA (392 mg, 1 mmol), glycine ethyl ester hydrochloride (396 mg, 2.82 mmol), *N*-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 403 mg, 1.63 mmol), triethylamine (Et_3N, 2.3 mL), and absolute EtOH (20 mL) were mixed in an Erlenmeyer flask and stirred manually, and the clear solution was heated in a domestic microwave oven for a period of 5–7 min at 70% power (total microwave output 650 Watts). In most cases, irradiating in intervals was appropriate. After cooling, the residual ethanol was evaporated and the reaction mixture dissolved in ethyl acetate and washed with water, 0.5 N NaOH, and 0.5 N HCl. The organic phase was washed with water again, dried (using anhydrous Na_2SO_4), and concentrated under reduced pressure to yield the ethyl ester of ursodeoxycholyl glycine, which was subsequently hydrolyzed to free acid by dissolution in EtOH (10 mL), treatment with potassium carbonate (10% aqueous solution, 7 mL), and irradiation for a total of 4 min in the microwave oven. The final solution was then poured into ice water (100 mL) with vigorous stirring, and acidified with 0.5 N HCl. The white solid which precipitated was collected by filtration and dried to yield **2a** (391 mg, 87%). By TLC, the R_f was 0.21 with solvent system A, and 0.54 with solvent system B. In ^{1}H NMR, δ = 7.03 (1H, *tr*, *J* = 4 Hz, N*H*), 3.26 (2H, *d*, *J* = 4 Hz, NH-CH_2) (15).

*Tauroursodeoxycholic acid (TUDCA) (***2d***, Scheme 2).* A mixture of UDCA (73.0 mg, 0.2 mmol), EEDQ (62.0 mg, 0.25 mmol), taurine (50 mg, 0.4 mmol), *N*-methylmorpholine or Et_3N (0.35 mL), and absolute EtOH (6.0 mL) were irradiated for 3.0 min in an Erlenmeyer flask in a commercial microwave oven. After cooling, the reaction mixture was poured slowly into chilled anhydrous ether (25 mL). The suspension was stirred at 5°C for 30 min and then filtered. The solid was dissolved in chloroform (10 mL) and filtered to remove unreacted taurine. The filtrate was poured into chilled anhydrous ether (25 mL). TUDCA was filtered and dried under vacuum (66.9 mg, 67%). By TLC, R_f was 0.80 with solvent system A. In ^{1}H NMR, δ = 7.67 (1H, *tr*, N*H*), 3.28 (2H, *q*, NH-CH_2), 2.50 (2H, *tr*, CH_2-SO_3). ESI–MS (in the negative ion mode) $[M - H]^-$ = 498 and other polymeric ions, $[2M - H]^-$ = 997, $[2M - 2H + Na]^-$ = 1019 and $[3M - 3H + 2Na]^-$ = 1540, were also observed (15).

*Tauroursocholic acid (***2e***, Scheme 2).* Tauroursocholic acid

TABLE 1
Chemical Shifts of the Side Chain for the Bile Acid Conjugates and Their Respective *N*-Nitroso Derivatives

Bile acid[a]	Side chain	Chemical shift
UDCA	R–CO–NH–CH_2–COOH	7.03 (*tr*, NH, *J* = 4 Hz); 3.26 (*d*, NH–CH_2, *J* = 4 Hz)
UDCA	R–CO–N(NO)–CH_2–COOH	4.42 [*s*, N(NO)–CH_2]; 13.25 (*s*, COOH)
UDCA	R–CO–NH–CH_2–CH_2–SO_3H	7.67 (*tr*, NH); 2.50 (*tr*, CH_2–SO_3H); 3.28 (*q*, NH–CH_2)
UDCA	R–CO–N(NO)–CH_2–CH_2–SO_3H	3.93 [*tr*, N(NO)–CH_2, *J* = 8 Hz]; 2.36 (*tr*, CH_2–SO_3H)
UCA	R–CO–NH–CH_2–COOH	8.09(*tr*, NH, *J* = 6 Hz); 3.70 (*d*, NH–CH2, *J* = 6 Hz); 12.4 (*s*, COOH)
UCA	R–CO–N(NO)–CH_2–COOH	4.42 [*s*, N(NO)–CH_2]
DCA	R–CO–NH–CH_2–COOH	8.10 (*tr*, NH, *J* = 6 Hz); 3.70 (*d*, NH–CH_2, *J* = 6 Hz); 12.45 (*s*, COOH)
DCA	R–CO–N(NO)–CH_2–COOH	4.41 [*s*, N(NO)–CH_2]; 12.0 (*s*, COOH)

[a]DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid); UCA, ursocholic acid (3α,7β,12α-trihydroxy-5β-cholan-24-oic acid); and UDCA, ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid).

was prepared from UCA in 65% yield following the same procedure that was used for the preparation of TUDCA. In TLC, R_f was 0.60 with solvent system A. ESI–MS (in the negative ion mode) $[M - H]^-$ = 514 and similar clusters of polymeric ions as in TUDCA were observed: $[2M - H]^-$ = 1029, $[2M - 2H + Na]^-$ = 1051 and $[3M - 3H + 2Na]^-$ = 1588.

N-*Nitroso glycoursodeoxycholic acid (NOGUDCA) (***3a***, Scheme 3)*. In view of the hazardous nature of *N*-nitroso compounds, all work was carried out in an efficient fume hood. GUDCA (100 mg, 0.22 mmol) was dissolved in concentrated glacial acetic acid (2 mL) in a three-necked round-bottom flask. The flask was kept at 2–4°C in an ice bath, and the contents were stirred by magnetic stirrer. In a separate round-bottom flask, sodium nitrite (10 g) was allowed to react with concentrated hydrochloric acid (15 mL), added dropwise through a separatory funnel. The liberated nitrous acid (HNO_2) gas was passed through a rubber tube and was bubbled into the reaction flask containing the bile acid conjugate dissolved in glacial acetic acid. After all the nitrous acid gas had passed (*ca*. 15 min.) and a green color persisted, TLC was taken while the mixture was kept frozen. The reaction was terminated if the TLC showed complete disappearance of the GUDCA (R_f = 0.21, solvent system D) and therefore maximal formation of the *N*-nitroso derivative (R_f = 0.44, solvent system A).

We have chemically synthesized (15) the following *N*-nitroso derivatives (Scheme 3) of 7β-hydroxylated bile acid conjugates: (i) *N*-nitrosoglycoursodeoxycholic acid (NOGUDCA), (ii) *N*-nitrosoglycoursocholic acid (NOGUCA), (iii) *N*-nitrosotauroursodeoxycholic acid (NOTUDCA), and (iv) *N*-nitrosotauroursocholic acid. In addition, *N*-nitrosoglycodeoxycholic acid (NOGDCA), a derivative of the secondary bile acid that has recently been widely implicated in colon cancer (42,43), was synthesized.

The structure and stereochemistry of the compounds in Scheme 3 were confirmed by high-resolution proton NMR, FAB–MS, and ESI–MS studies. The ^1H NMR spectra of the *N*-nitroso bile acid conjugates as well as of their respective bile acid conjugates were obtained. Table 1 contains a summary of the significant chemical shifts of the side chain. The major part of the spectrum, δ 0–2.2, contains peaks corresponding to the steroid backbone and remains essentially unchanged for all the molecules.

For all of the *N*-nitroso bile acid conjugates prepared, MS indicated that the first fragment involved rearrangement and the loss of N_2. The FAB–MS of the *N*-nitroso glyco-bile acid conjugates were taken in NaCl in the positive ion mode, and consequently they showed sodiated molecular ions at *m/z* 501 = $[M + Na]^+$ for NOGUDCA and NOGDCA. A molecular ion peak at *m/z* 517 = $[M + Na]^+$ was observed for NOGUCA. In the ESI–MS negative ion mode, the *N*-nitrosotauro-bile acid conjugates showed $[M - H]^-$ peaks at *m/z* 542.8 for *N*-nitrosotauroursocholic acid and *m/z* 527.1 for NOTUDCA. Table 2 shows the first three major mass spectral peaks and the fragmentation pattern for these compounds. Especially important are the carboxymethyl and 2-sulfoethyl moieties from the side chain of the *N*-nitrosated bile acid con-

TABLE 2
Major Mass Spectral Peaks for the *N*-Nitroso Derivatives[a]

(FAB–MS) positive ion mode				
Glyco-derivative	MW	$[M + Na]^+$	–N_2,–H	–CH_2–COOH
NOGUDCA	478	501.4	472.4	413.4
NOGUCA	494	517.4	488.4	429.4
NOGDCA	478	501.3	472.4	413.4
(ESI–MS) negative ion mode				
Tauro-derivative	MW	$[M - H]^-$	–N_2	–CH_2–CH_2–SO_3
NOTUDCA	528	527.1	499.1	391.1
NOTUCA	544	542.8	515.3	407.1

[a]FAB–MS, fast atom bombardment–mass spectrometry; ESI–MS, electrospray–mass spectrometry; NOGUDCA, *N*-nitrosoglycoursodeoxycholic acid; NOGUCA, *N*-nitrosoglycoursocholic acid; NOGDCA, *N*-nitrosoglycodeoxycholic acid; NOTUDCA, *N*-nitrosotauroursodeoxycholic acid; and NOTUCA, *N*-nitrosotauroursocholic acid.

SCHEME 4

jugates because of their roles in the alkylation of DNA.

Decomposition of N-*nitroso glyco-bile acid conjugates (Scheme 1).* The glyco-compounds, NOGUDCA, NOGUCA, and NOGDCA decomposed between pH 6 and 9 in aqueous buffer solutions, indicating a $t_{1/2}$ of 5–7 h on average at zero buffer concentration and neutral pH. Under the same conditions, NOTUDCA indicated a much longer $t_{1/2}$ of approximately 15–17 h. The kinetics of decomposition were found to be first-order over three half-lives. The decomposition that we have observed is significantly slower than the $t_{1/2}$ of 2–4 h reported for NOTC and NOGC (15,16). We plan to use four compounds for biological studies, namely (i) NOGUDCA, (ii) NOGUCA, (iii) NOTUDCA, and (iv) NOGDCA. The behavior of NOTUDCA and NOGC in forming carboxymethyl electrophilic alkylating agents which bind with DNA nucleotides is consistent with its expected decomposition as an *N*-nitrosamide (Scheme 1) (15,16,19,26,29,30).

Mechanism of decomposition of some alkanediazoates. Hydrolysis of N-*nitroso bile acid conjugates, TUDCA and CA with cholylglycine hydrolase (Scheme 4).* The complete nitrosation of TUDCA and GCA was confirmed by TLC analysis, using the solvent system chloroform/methanol 40:3 (vol/vol). Further resolution of the reaction mixture on a preparatory TLC plate indicated *E*- and *Z*-rotomeric populations in ratio of 60:40. The *E*- and *Z*-rotomers depend upon the rotation of the NO molecule in relation to the ketonic group of the conjugated bile acid (Scheme 4). The final product was recovered by washing with methanol and precipitating with ether. The precipitate was filtered and stored in the freezer to minimize decomposition.

As the *N*-nitroso bile acid conjugates decompose, several carcinogenic species are generated (Schemes 1,4). First, an alkyl diazoate is formed which is subsequently protonated. This species then loses nitrogen (N_2) and forms either glycolic acid (from NOGCA) or isethionic acid (from NOTUDCA). Another species which exists is a diazoglycolic acid or diazoisethionic acid, corresponding to NOGUDCA and NOTUDCA, respectively. These decomposition products were neatly isolated by using preparatory TLC and identified and characterized by chemical ionization mass spectroscopy. Mass spectrum results showed peaks at 126, 137, and 154 *m/z* corresponding to the decomposition fragments isethionic acid, diazoisethionic acid, and the protonated diazoate intermediate, respectively. The mass spectrum analysis displayed the highest abundance for the isethionic acid (a urinary metabolite of taurine) peak at 126 *m/z*. These electrophilic species described above are implicated in the alkylation of DNA which results in the tumorigenesis associated with colorectal carcinomas. In conjunction with these products, the free bile acid UDCA was also formed. Successful synthesis of the *N*-nitrosated bile acids enabled a series of decomposition assays which aided in the elucidation of the specific mechanism implied in the decomposition pathway resulting in the aforementioned products.

The compound NOTUDCA was subjected to the enzymatic cleavage reaction with cholylglycine hydrolase, a bac-

terial enzyme (44,45) which cleaved the *N*-nitrosated bile acid conjugate at the peptide bond. TLC analysis further revealed that this assay generated several decomposition products. This result was further substantiated when products with similar R_f values were generated in an alkaline cleavage assay conducted with lithium hydroxide in isopropranol. Both assays cleaved the NOTUDCA at the peptide bond generating the free bile acid UDCA and three major decomposition products which were confirmed by chemical ionization mass spectroscopy.

The results obtained in these studies, specifically the identification of the diazoate intermediate as revealed by the mass spectrum peak at 154 *m/z,* further substantiate that the decomposition route follows a mechanism which generates diazonium compounds (18,19,46). Following decomposition, the diazo compounds lose N_2 to produce isethionic acid in the highest abundance due to its high stability.

DISCUSSION

The hypothesis that the *N*-nitroso bile acid conjugates may be involved in carcinogenesis is based on several well-founded premises. Although they have not yet been detected *in vivo* owing to a lack of appropriate methodology, our work suggests that they can be formed. The etiology of stomach cancer is linked to a diet rich in nitrate or nitrite, consumption of smoked foods, and damage to the gastric mucosa by irritants such as salts (47). According to a gastric cancer model proposed by Correa (26), a rise in gastric pH allows bacteria that convert nitrate to nitrite to flourish. Nitrite can then participate in nitrosation reactions in acidic solutions to form *N*-nitroso compounds. A characteristic of the conjugated bile acids is a decreased pK in comparison to the free bile acids and thus increased solubility in acidic solutions (48). The reaction of the bile acid conjugates with nitrite (or other nitrosating agents) is more likely to take place in the acidic environment of the stomach when certain clinical states cause the reflux of bile acids into the stomach from the intestine or bile duct (49). In the unoperated stomach, these states include reflux gastritis and chronic gastritis (50). Carcinoma in the stomachs of patients who have undergone Billroth II gastrectomies has been associated with enterogastric reflux (51).

It is essential to note that CA and chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid, CDCA) are two primary bile acids in the human gastrointestinal tract, and many other stereoisomeric and ketonic products are formed by bacterial action (21–23,52,53). Once in the hepatocyte, primary bile acids are conjugated with glycine or taurine to form the corresponding *N*-acyl conjugates (21–23). As conjugates they are secreted from the hepatocyte into the bile and with the bile they enter the intestine. In the distal small intestine and in the colon, these conjugates are converted by bacterial enzymes into the corresponding secondary bile salts by initial deconjugation, followed by 7α-dehydroxylation (52). This process in the case of CA leads to the formation of deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid, DCA), and lithocholic acid (3α-monohydroxy-5β-cholan-24-oic acid) results from CDCA. Most of the lithocholic acid that is formed is excreted from the body by a sulfation process. DCA, however, often becomes a major bile acid in the enterohepatic circulation in human adults because 7-rehydroxylation does not occur (21,23). While the mechanism responsible for the putative cancer-promoting effects of bile acids is not defined, the suspect causative bile acids such as DCA and CDCA are cytotoxic to colon epithelial cells (54,55) and promote chemically induced colon cancer (56,57). DCA has been implicated in the promotion of adenoma growth and polyp formation, although the mechanism by which this occurs is not clear (42,43,58–60).

In addition, numerous other products, mainly epimers of the major bile acids having 3β, 7β, and 12β stereochemistry, may also be produced by the bacterial action in the distal ileum or colon (52,53). Bile acids of the urso family, with a 7β configuration, include ursocholic acid (3α,7β,12α-trihydroxy-5β-cholan-24-oic acid, UCA) and UDCA which are 7β epimers of CA and CDCA, respectively. UDCA, present in bear's bile, is of special interest because it has been approved by the U.S. Food and Drug Administration as an efficient gallstone solubilizer (Actigall®, Ursodiol) and is being consumed daily in large amounts by those suffering from gallstone disease and primary biliary cirrhosis (61–64). Recently, the taurine conjugate of UDCA has also been reported to have superior cholesterol-solubilizing properties (61–63). Although the toxicity of UDCA has been observed to be minimal thus far, the long-term consequences of therapy with this drug or its conjugate remain to be discovered.

N-Nitroso bile acid conjugates of the 3β, 7β, and 12β stereochemistry may form endogenously, and their end products upon decomposition may be very potent alkylating agents. *N*-Nitroso compounds occur in normal gastric juice (65). However, in subjects who were prone to develop gastric cancer or had undergone Billroth I or II operations these compounds were found to be increased (65). Analytical methods cannot identify the specific endogenous compounds until they have been purified, identified, and biologically assayed (33,34,65).

It is essential to keep in mind that a large pool of bile acid conjugates, 10–40 g, travels through the enterohepatic circulation daily (23), including large amounts of the primary bile acids, CA and CDCA, and of the secondary bile acid, DCA. In addition, UDCA is present in large amounts in the bile of patients taking this compound as medication. If *N*-nitroso amides from these bile acids do form in the stomach, they may flow into the intestine with the rest of the semiliquid mass from the stomach. Because the half-lives of the *N*-nitroso bile acid conjugates are relatively long, and the conjugates are stable under the conditions (15) that might be encountered in the gastrointestinal tract, it is likely that they can travel through the intestines. While many of the bile acid conjugates are deconjugated in the intestine by bacteria, it is important to study the effects of intestinal enzymes such as cholylglycine hydrolase on the *N*-nitroso bile acid conjugates

(44). It has been found that when TUDCA is conjugated with a "cyclopropylog," the presence of the cyclopropyl ring adjacent to the amide bond makes the molecule resistant to degradation (66). The nitroso group on the other side of the amide bond may also have an interesting effect. Indeed, when the compounds NOTUDCA and NOTCA were subjected to the enzymatic cleavage reaction with cholylglycine hydrolase (44,45), a bacterial enzyme (Scheme 4), it cleaved the *N*-nitrosated bile acid conjugate at the peptide bond resulting in the formation of three major decomposition products which were confirmed by chemical ionization mass spectroscopy. These experiments substantiated that the decomposition route follows a mechanism which generates diazonium compounds. These further decompose to give electrophilic species, which react with DNA to give rise to methyl adducts (Scheme 4).

Recently several papers from Vogelstein's laboratory (67–69) have delineated the mechanism of colon cancer as a distinct type of genetic disease in which not one, but several, mutations are required. According to the authors, the development of a colorectal carcinoma involves the inactivation of at least three tumor suppressor genes, p53 (70,71), APC (Adenomatous Polyposis Coli), and DCC (Deleted in Colorectal Carcinomas) (69–71). Each mutation creates a wave of cellular multiplication associated with gradual increase in malignancy. Approximately 5 to 10% of colorectal cancers are hereditary and classified as hereditary nonpolyposis colorectal cancer (70). One of the genes involved in hereditary nonpolyposis colorectal cancer is hMLH1, a human mismatch repair gene, and a mutation of hMLH1 can disrupt the gene product (70,71). A variety of factors may be responsible for the mutations, although mutations caused by endogenously formed compounds are a possibility that requires further investigation. ^{14}C-NOGC has been found to form carboxymethylated adducts with the nitrogenous bases guanine and adenine in calf thymus DNA *in vitro*. The detection of carboxymethylated nucleic acid bases and proteins appears to be a useful way of monitoring endogenous formation of NOGC and related compounds (34). It is urgent to develop such methodology so that patients with cancers of the stomach, liver, or colon may be screened (72,73) for the *N*-nitroso bile acid conjugates.

There are many avenues for further research on the topic of *N*-nitroso bile acid conjugates. We are in the process of synthesizing *N*-nitroso derivatives of CA, CDCA, and lithocholic acid having 3β, 7β, and 12β stereochemistry. We will then study the chemical interactions of the molecules with calf thymus DNA. DNA adduct formation will be analyzed by spectroscopic methods including proton magnetic resonance, differential Fourier transform infrared spectroscopy, and circular dichroism. Polymerase chain reactions will be employed to replicate the adducted calf thymus DNA, and the mutation of this DNA in comparison to pure calf thymus DNA will be observed by Southern blot.

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Synthesis and Molecular Modeling: Related Approaches to Progress in Brassinosteroid Research

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ABSTRACT: In the field of brassinosteroids, which are potent plant growth regulators, we have developed a quantitative structure–activity relationship study to develop knowledge from a structural point of view and to find out new requirement definitions. This will help identify other suitable active brassinosteroid derivatives with a good activity/synthetic cost ratio for further application in agriculture. The methodology used to achieve this goal represents a multidisciplinary study involving synthesis, molecular modeling calculations, and bioactivity evaluation. The influence of different molecular properties in the bioactivity of a set of synthetic compounds (i.e., molecular electrostatic potential and the ability to form H bonds) is discussed. The molecular electrostatic potential is expressed in terms of the electrostatic Carbó similarity index (CI) between brassinolide (**1**) and other brassinosteroids. We have found that the electrostatic charges of the functional groups play an important role in the description of the activity, as evidenced by its good correlation with the CI in most cases. Deviation from this rule could be explained by the H bonding abilities of some of these compounds, which we believe may play an essential role in binding to the natural receptors.

Lipids 32, 1341–1347 (1997).

Brassinosteroids are potent naturally occurring plant growth regulators widely spread in the vegetal kingdom. They have an exciting potential use in agriculture owing to their capability of improving crop yield and quality as well as minimizing environmental stress and herbicidal injury and controlling pathogenic diseases (1,2).

At least 36 natural brassinosteroids have been identified from different parts of plants: pollen, seeds, leaves, shoots, etc., brassinolide (**1**) being the most potent natural brassinosteroid (3). From a structural point of view, brassinosteroids are polyhydroxylated steroids differing in their functionalities and the stereochemistry present in the A and B rings and the side chain.

Brassinosteroids have been tested as plant growth-promoting hormones in more than 20 bioassays typical for phytohormones such as auxins, gibberellins, or cytokinins (1). From all of them, the rice lamina inclination test is one of the most specific for brassinosteroids, being widely used for activity evaluation (4,5).

Because of the interest of such compounds and the high synthetic cost of the most active brassinosteroids, the goal of our study was to find other suitable active derivatives, with a good activity/synthetic cost ratio for agricultural application. For this purpose, minimal structural requirements for brassinosteroid activity should be known.

Different qualitative structure–activity relationships have already been established from the activity data obtained in special bioassay systems (6,7). Although these relationships are more or less strictly dependent upon the bioassay used, it is generally accepted that the structural requirements for a high brassinosteroid activity are: (i) 2α,3α-diol in A ring, (ii) 7-oxalactone better than 6-ketone in B ring, (iii) A/B *trans* fused ring junction, (iv) a *cis* C_{22},C_{23} diol preferably with 22*R*,23*R* configuration, and (v) a C_{24} methyl or ethyl substituent. Nevertheless, a closer look into these requirements and into how they were established (only a limited number of brassinosteroid analogs with few structural modifications have been assayed) reveals that they are far from being general from two points of view.

First, the brassinosteroid functionalities cannot be considered independently, as shown in these requirements, since we have found that they are closely related. Figure 1 shows activity data for nine brassinosteroids, measured using our modified rice lamina inclination test (Brosa, C., Soca, L., and Terricabras, E., manuscript in preparation) based on the procedure developed by Takeno and Pharis (4). In agreement with the general rule, it can be observed that compounds with lactone in B ring (**2**, **3**, **6**, and **7**) elicit higher activity than their corresponding 6-ketone analogs (**4**, **5**, **8,** and **9**). (see Scheme 1 for structures). In the same sense, compounds with 22*S*,23*S* configuration at the side-chain diol (**3**, **5**, **7,** and **9**) are less active than the corresponding 22*R*,23*R* ones (**2**, **4**, **6,** and **8**), but the values strongly depend not only on the configuration of the diol at C_{22},C_{23} but also on the type of alkyl substituent at C_{24}. Thus, for a similar functionality in the skeleton, compounds with stigmastane side chain (**2** to **5**) are more active than those with an ergostane side chain (**6** to **9**) except for **7** which is more active than **3**. Moreover, with respect to con-

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Abbreviations: CI, electrostatic Carbó similarity index; 3D, three-dimensional.

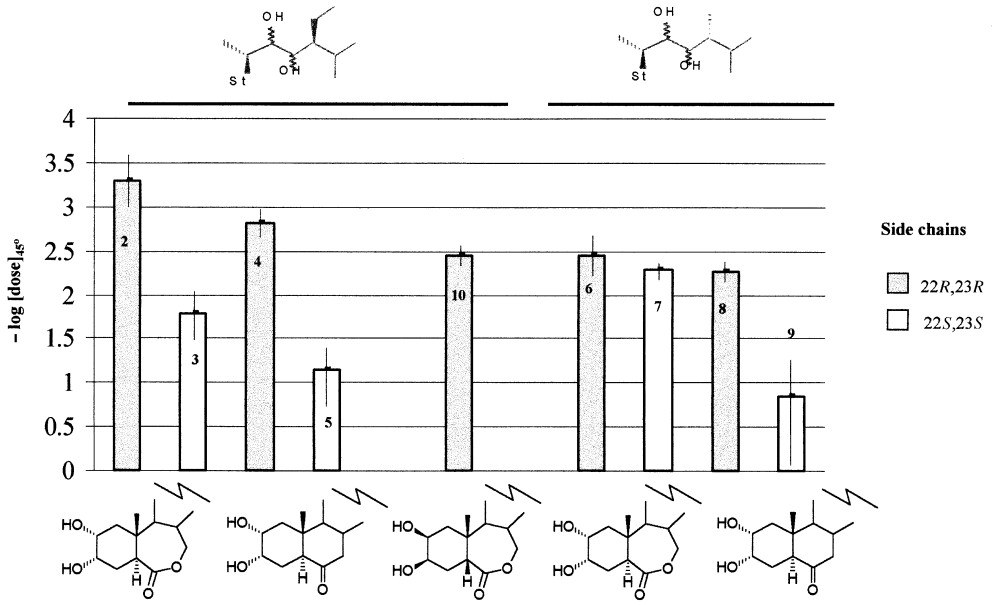


FIG. 1. Brassinosteroid activity in the rice lamina inclination test. Numbers on bars correspond to compounds in Scheme 1. Bars represent the activity value of each compound.

figuration of the diol, lactone **6** is less active than the 22*R*,23*R* ketone analog **4**, thereby not meeting the requirements. But when the configuration changes from 22*R*,23*R* to 22*S*,23*S* the activity also changes, and now becomes higher for **7** than for **5**. Therefore, these results clearly indicate the existence of a relationship between functionalities and thus a strong interdependence among requirements (ii), (iv), and (v). Furthermore, similar relationships are observed for the rest of the requirements.

The second point for which we believe that these requirements are far from being general is related to the brassinosteroids involved. The postulated requirements limit the scope of applicability to the functionalities involved in the tested set of brassinosteroids. For instance, they were developed without taking into account analogs having a 2β,3β diol and/or A/B *cis* junction. No brassinosteroids with these functionalities were examined. So, why were they not considered for the requirements if there was no proof of their activity? After synthesizing such compounds and evaluating their activity, we obtained a very high activity for **10**, with 2β,3β diol and A/B *cis* junction (Fig. 1) (8,9).

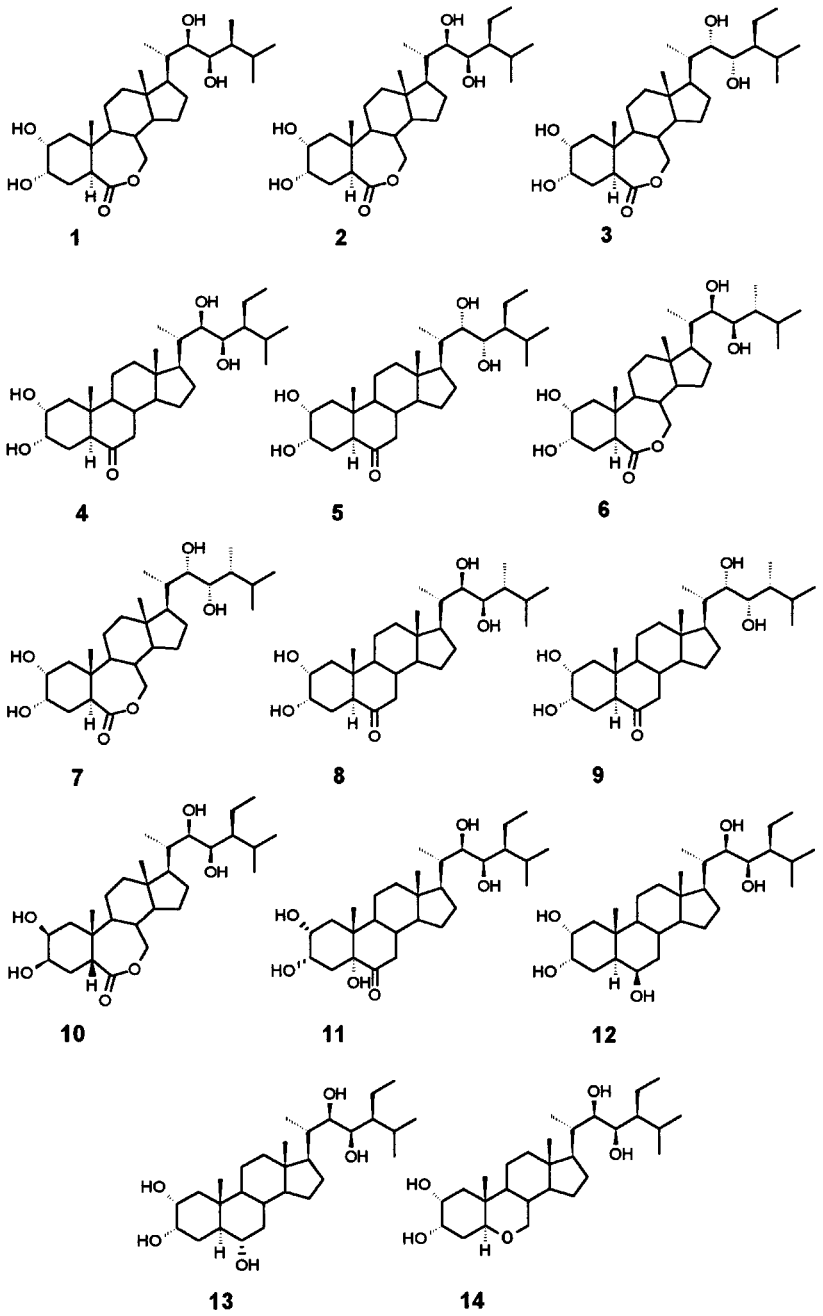
In short, the close relationship between the brassinosteroid functionalities and the high activity elicited by this new brassinosteroid **10** indicates the weakness of the requirements postulated in the literature for a high brassinosteroid activity. Thus, a more accurate way to define the structural requirements should be found.

In assuming that brassinosteroids may act at the molecular level through a mechanism similar to that of animal steroid hormones, a receptor/ligand complex which binds to nuclear or cytoplasmatic sites to regulate the expression of specific genes should be involved. Therefore, the active brassinosteroids should have a single defined three-dimensional (3D) "active conformation" which best fits the receptor or receptors. On this active conformation, the atoms directly involved in binding with the brassinosteroid receptor ought to have the same spatial situation in all active molecules. Thus, the more complementary is the active conformation of a defined brassinosteroid to the 3D structure of the receptor, the more active it should be. Since brassinolide (**1**) is the most active natural brassinosteroid found, we can assume that its active conformation will be the one that best fits to the receptor and that can be taken as reference. In this sense, a new way to define the structural requirements for a high brassinosteroid activity has been considered.

The strategy to achieve this goal involves molecular modeling techniques which also enable the establishment of a quantitative structure–activity relationship (QSAR). Apart from providing information about brassinosteroid receptor binding, the results will eventually be of help in the design of the most suitable brassinosteroids for agricultural applications. An analysis of the results obtained in the application of this approach is presented here.

MATERIALS AND METHODS

To establish a QSAR, a broad set of brassinosteroids having sufficient structural modifications, together with their corresponding strictly homogeneous activity data with statistical parameters, has been used. Different parameters have been calculated and their correlations with activity have been investigated. In Scheme 1 are presented the brassinosteroids mentioned in the present paper. Except for brassinolide (**1**), we have synthesized all of them (8–15). The first difficulty in this strategy is the lack of homogeneity and statistical parameters on the activity data obtained from the literature, so a bioassay was developed in order to derive strictly homogeneous activity data and their corresponding statistical para-



SCHEME 1

meters (Brosa, C., Soca, L., and Terricabras, E., manuscript in preparation).

In following this methodology, a modified active analog approach has been used to obtain the active conformer for each brassinosteroid. This study involves a conformational analysis, a 3D structure comparison, and a conformer alignment (16). The conformational analysis has been carried out by a systematic tree analysis and corroborated by molecular dynamics. For the 3D structure comparison, a program has been developed to calculate three different similarity indexes among the conformers of each compound: the square residue (SQR), the root mean square (RMS), and the molecular overlay volume (MOV). In the last step, the structure alignment was performed by optimization of the electrostatic Carbó similarity index (CI) (17) calculated using the ASP program (18).

The ability to form H bonds has been calculated with the GRID program (19). The preliminary results are given using water as a probe.

RESULTS AND DISCUSSION

The CI was selected to align the molecules since it is a measure of the similarity of electrostatic potential between two molecules. Moreover, these kinds of electrostatic interactions

TABLE 1
Brassinosteroid Electrostatic Carbó Similarity Index (CI) and Rice Lamina Inclination Test Activity

Compound[a]	CI[b]	–log (dose)$_{45°}$
1	1	5.93
2	0.82	3.29
4	0.76	2.80
6	0.97	2.45
10	0.76	2.45
7	0.72	2.27
8	0.81	2.27
3	0.75	1.78
11	0.76	1.53
12	0.58	1.36
5	0.74	1.14
13	0.48	1.13
9	0.52	0.84
14	0.67	0.47

[a]Compound numbers correspond to structures presented in Scheme 1.
[b]The value of the CI depends on the alignment. The confidence limit is ±0.05.

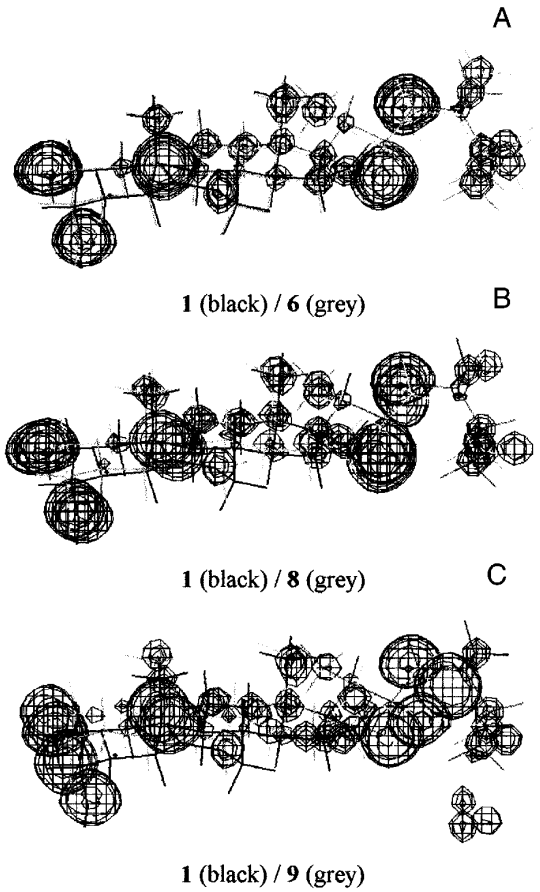


FIG. 2. Overlaid molecular electrostatic potential maps at –30 KT level between brassinolide (**1**) (in black) and three brassinosteroids (**6**, **8**, **9**) (in grey). The electrostatic Carbó similarity index/activities for these compounds are: (A) 24-epibrassinolide (**6**), 0.97/2.45; (B) 24-epicastasterone (**8**), 0.81/2.27; and (22*S*, 23*S*) 24-epicastasterone (**9**), 0.54/0.84.

are very important for compound binding to a receptor. Thus, the more similar a compound is to brassinolide (1), the higher the CI.

Table 1 shows the CI and the activity data for some of the brassinosteroids studied. In general, activity correlates with the CI and is higher when the CI is over 0.75, although there are some exceptions.

For example, Figure 2 shows how the electrostatic potential contour map at –30 KT for brassinolide (**1**) overlays with that of **6** (Fig. 2A), **8** (Fig. 2B), and **9** (Fig. 2C). In agreement with its high activity, all the electrostatic potential sites for 24-epibrassinolide (**6**) (in grey) overlay perfectly with brassinolide (**1**) (in black), with a very high CI (0.97). In the case of 24-epicastasterone (**8**), which shows lower activity than **6**, only the B-ring region does not overlay well with the lactone of brassinolide (**1**), decreasing its CI (0.81). Finally, in the case of (22*S*,23*S*) 24-epicastasterone (**9**), which displayed very low activity, neither the region of the A ring and side chain nor the region of the B ring overlays well with brassinolide (**1**), as is indicated by its very low CI (0.52). These compounds are a good example of how the activity decreases along with CI.

Furthermore, a similar relationship is observed for compounds **2**, **4**, **12**, and **13**, differing only on the B ring (Fig. 6A).

Note that 28-homobrassinolide (**2**), 28-homocastasterone (**4**), 24-epibrassinolide (**6**), and 24-epicastasterone (**8**) fulfill the requirements postulated in the literature, although they show different activities, ranging from 3.29 to 2.27 in a logarithmic scale. These activity differences are better explained through their CI. Thus, it seems that this similarity index (CI) is a more precise way to establish the requirements. Nevertheless, in some cases the activity cannot be explained by the CI. Thus, in the case of the lactone **10**, the activity is higher than expected for a CI of 0.76. Conversely, the activity of the 6-oxa analog **14** is lower than expected for a CI of 0.67.

Moreover, the three compounds represented in Figure 3 (**4**, **10**, and **11**) have a very similar CI but they elicit different activities. Looking at the overlaid maps (Fig. 3), one can observe that, for **10** (Fig. 3A), although having a 2β,3β-diol and A/B *cis* junction, all the electrostatic potential sites of this compound overlay very well with the ones of brassinolide (**1**) except for the region near the hydroxy group at C_2. This could indicate the minor importance of this hydroxy group to the activity. Concerning the hydroxyketone **11** (Fig. 3B), the regions corresponding to the diols of the side chain and A ring are larger than those of brassinolide (**1**). Therefore, although this similarity index proved to be better than the previous requirements, it is not enough to set up a brassinosteroid CI-related activity index. Other factors may be involved.

In considering the initial hypothesis, if brassinosteroids acted through a mechanism similar to that of animal steroid hormones, the brassinosteroid receptor complex could involve H bonds between the protein residues and the steroid. Thus, if the region where the probability to form H bonds and the interaction energy for these compounds was found, it should be possible to discriminate between them and, moreover, to gain more information about the mechanisms of brassinosteroid receptor interaction.

The GRID methodology (19) has been used to calculate

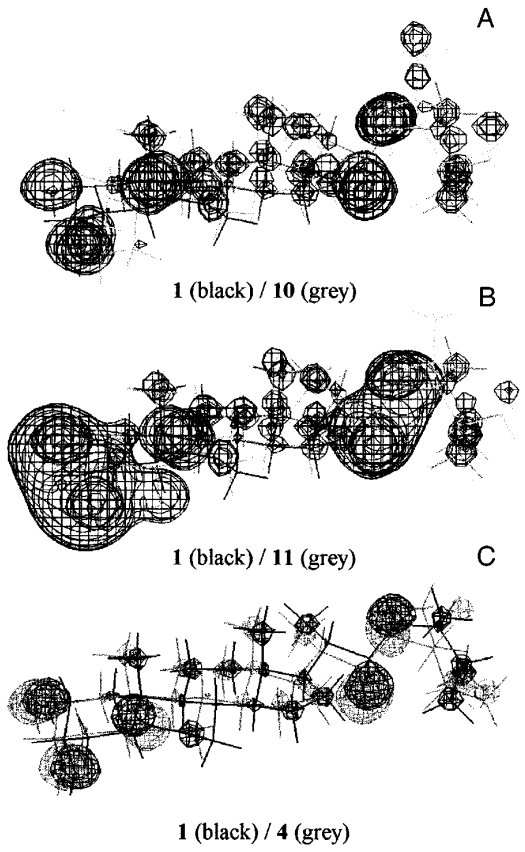


FIG. 3. Overlaid molecular electrostatic potential maps at –30 KT level between brassinolide (**1**) (in black) and three brassinosteroids (**4**, **10**, **11**) (in grey). The electrostatic Carbó similarity index/activities for these compounds are: (A) **10** 0.76/2.45; (B) **11** 0.76/1.53; (C) 28-homocastasterone (**4**), 0.76/2.80.

the energy interaction between the brassinosteroid and a probe that simulates an interaction by the H bond. On this preliminary calculation, water has been chosen as probe owing to its capability to act both as acceptor and donor of H bonds.

Figure 4 shows the GRID map at a –4 kcal/mol for brassinolide (**1**); the areas in white represent the highest probability to form H bonds.

Figure 5 shows the GRID map junctions only for the A and B rings between brassinolide (**1**) and **4**, **10**, and **11**, the same three compounds whose electrostatic potential maps are shown in Figure 3. The junction (in white) represents the common area between brassinolide (**1**) and each compound with a higher probability to form H bonds. As it has been indicated, all these compounds have the same CI but elicit very different activities. From these pictures, we see that, whereas the area corresponding to the hydroxy groups on the A ring for **4** and **11** is similar to that of brassinolide (**1**), there is only a small area on this region in **10**. This is in agreement with what was observed on the electrostatic potential map of **10** (Fig. 3), where the hydroxy group at C_2 was far more distant. If one takes into account the junction on the B ring, one observes that **4** presents a hole in the center and that its activity falls from 5.93 for brassinolide (**1**) to 2.80. This area becomes smaller for the hydroxyketone **11** and the activity falls again to 1.53. Moreover, in this case, another factor could decrease the ability of H bonding with the receptor owing to its capability of forming an intramolecular H bond between the two hydroxy groups at C_3,C_5. This phenomenon is observed in the active conformation found for **11** (16). Therefore, the size and shape of the area where the probability to form H bonds is higher seem to be activity-related. For the lactone **10**, two fea-

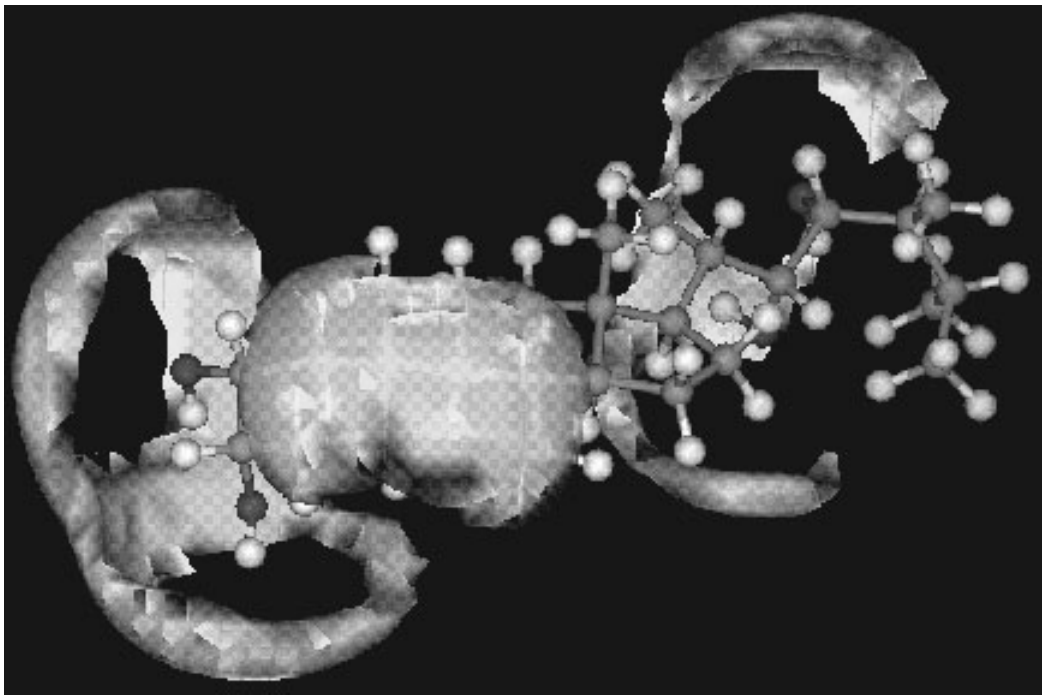


FIG. 4. GRID map for brassinolide (**1**) using a water probe at –4 kcal/mol.

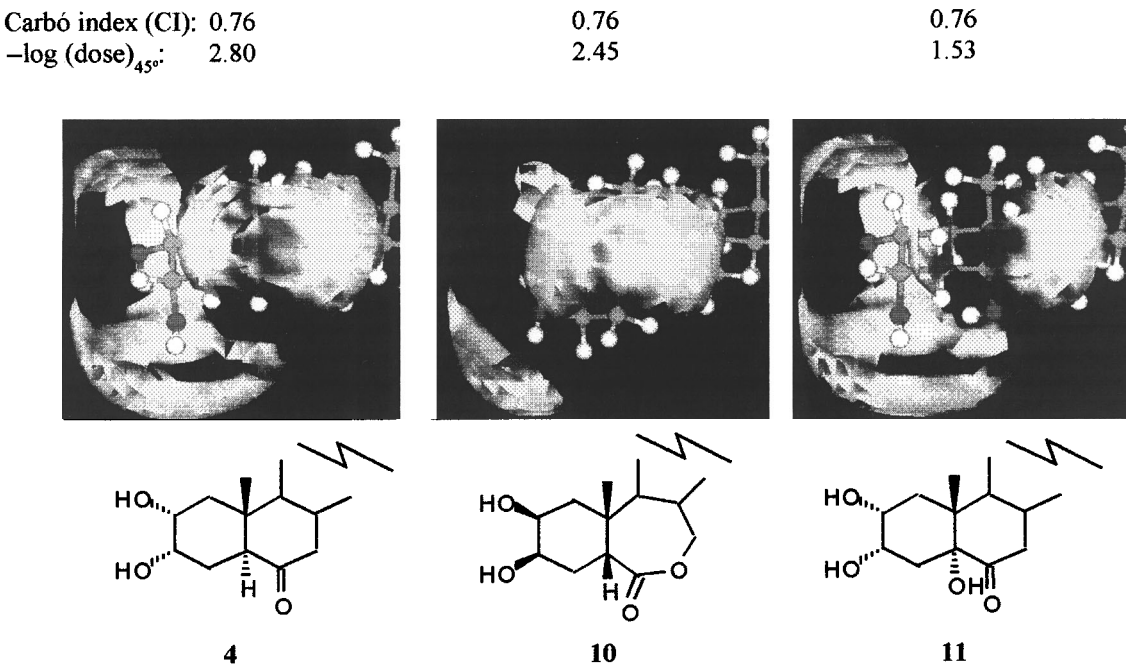


FIG. 5. GRID map surface junction for water probe between brassinolide (**1**) and three brassinosteroids (**4**, **10**, **11**). See Scheme 1 for structures **4**, **10**, and **11**.

tures of the molecule seem to compensate each other. On one hand, the area of the B ring is highly similar to brassinolide (**1**), so the activity should be higher than elicited; on the other hand, there is only a small common area for the hydroxy group at C_3, which forces its activity to decrease. Therefore, qualitatively one can assume that the ability to form H bonds

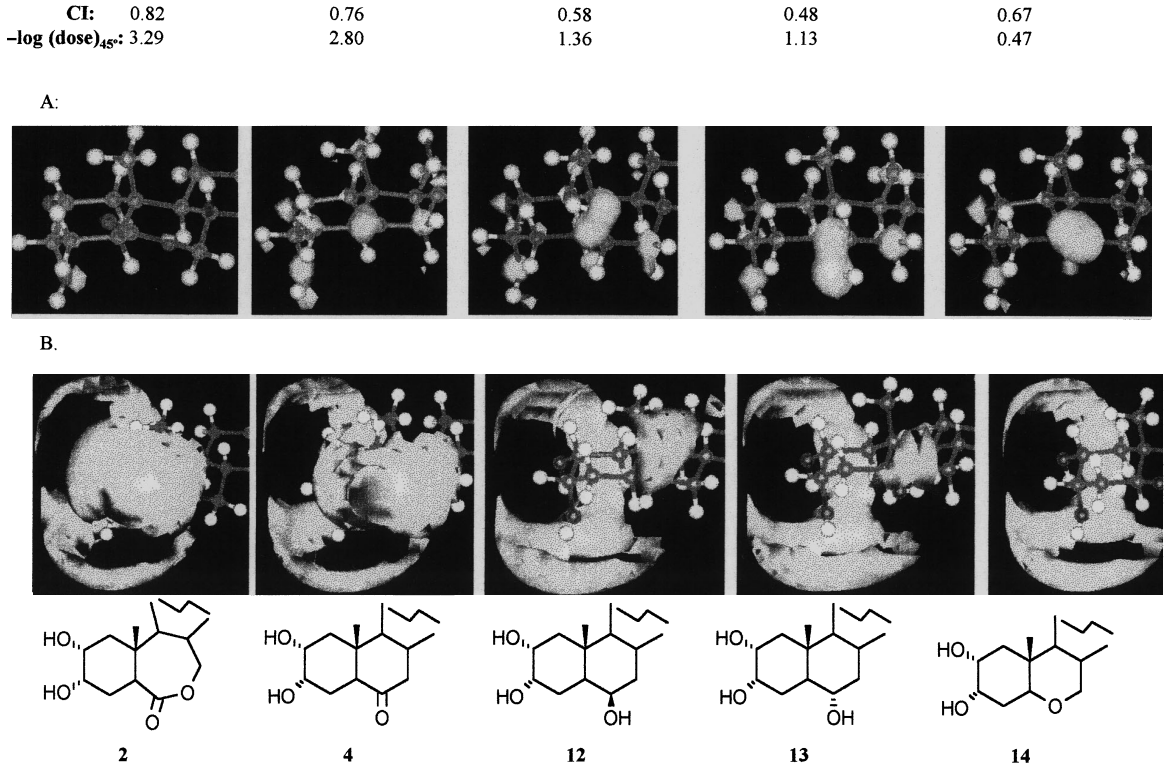


FIG. 6. (A) Surface difference in molecular electrostatic potential between brassinolide (**1**) and five brassinosteroids (**2**, **4**, **12**, **13**, **14**). (B) GRID map surface junction for water probe between brassinolide (**1**) and five brassinosteroids (**2**, **4**, **12**, **13**, **14**). See Scheme 1 for structures. See Figure 5 for abbreviation.

to the receptor could be suitable to describe the activity.

The 6-oxabrassinosteroid **14** is another example on how the ability to form H bonds is activity-related. Thus, on Figure 6A, the surface difference of molecular electrostatic potentials (in white) for A and B rings, between brassinolide (**1**) and 5 analogs differing only in B ring (**2**, **4**, **12**, **13**, and **14**) is shown. Qualitatively, one can observe that the white area, which is the difference between the electrostatic potential map for brassinolide (**1**) and each compound, increases and the activity decreases progressively in all cases as well as with the CI, except for the 6-oxabrassinosteroid **14**, for which its CI is higher than expected. An explanation for this exception can be found, again bearing in mind the ability of this group to form H bonds.

Looking at the surface junction of GRID maps between brassinolide (**1**) and the same five analogs already mentioned (**2**, **4**, **12**, **13**, and **14**) (Fig. 6B), one can see that the area on B ring, where the probability to form H bond is higher, gradually decreases in the series from **2**, up to disappearance for **14**. Qualitatively there is a very close relationship between this area and the activity. So, the impossibility of forming H bond on the B ring of the 6-oxa analog **14** is in agreement with its very low activity even with a CI higher than expected.

These results are the first reported evidence of the limit of scope of applicability and the weakness of the requirements so far postulated for a high brassinosteroid activity. In this sense, our new brassinosteroids having 2β,3β-diol and A/B *cis* ring junction (**10**), hydroxyketone (**11**), 6-hydroxy (**12** and **13**) or 6-oxa (**14**) functionalities, which had not been considered in these requirements, have shown activity as plant growth regulators and in some cases have even shown a high activity.

Moreover, in our aim to find new requirements definition, we found that the electrostatic charges of the functional groups play an important role in the description of the activity as evidenced by its good correlation with the CI in most of the cases studied. The differences observed in some of them could be explained by their ability to form H bonds. This could be one of the ways through which brassinosteroids interact with the receptor or receptors.

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